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Le rôle de l'axe CD40L/CD40/NF-κB dans la fonction plaquettaire

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Résumé

Le CD40 ligand (CD40L) est une molécule thrombo-inflammatoire qui prédit des événements cardiovasculaires. Les plaquettes constituent la principale source du CD40L soluble (sCD40L) dans la circulation, avec la capacité d'influencer la fonction des plaquettes par l'intermédiaire de sa liaison à ses récepteurs plaquettaires : CD40, $\alpha_{IIb}\beta_3$ et $\alpha_5\beta_1$. Nous avons précédemment démontré que la stimulation des plaquettes humaines avec le sCD40L induit une activation du *nuclear factor kappa B* (NF-κB), qui pourra jouer un rôle non génomique en amorçant les plaquettes. En effet, l'amorçage des plaquettes par le sCD40L augmente fortement l'activation et l'agrégation plaquettaire en réponse à des doses sous-optimales d'agonistes. Cependant, l'implication des différents récepteurs dans l'amorçage des plaquettes suite à l'activation du NF-κB par le sCD40L demeure inconnue. De plus, le *transforming growth factor-B (TGF-B)-activated Kinase* (TAK1), un régulateur majeur de l'inflammation, est activé par le sCD40L dans les cellules nucléées, et pourra ainsi être impliqué dans la signalisation sCD40L/NF-κB plaquettaire. D'autre part, le sCD40L se trouve en quantité élevée dans la circulation sanguine des patients coronariens. De plus, le traitement antiplaquettaire à l'Aspirine (ASA) est inefficace chez certains patients et son efficacité est réduite chez les patients à hauts risques d'évènements coronariens, ce qui augmente leur risque de sur-activation plaquettaire et de développer une thrombose. Des taux sanguins élevés de médiateurs thrombo-inflammatoires, tels que le sCD40L, peuvent expliquer de telles variabilités. Ainsi, ce projet a été entrepris dans le but d'élucider l'impact de l'axe sCD40L/NF-κB sur la fonction plaquettaire et sa modulation par l'ASA.

Dans le premier volet de mon projet, nous avons identifié les récepteurs de sCD40L impliqués dans l'activation du NF-κB plaquettaire, leur signalisation en aval et leur implication dans l'agrégation plaquettaire. Nous avons montré que les plaquettes exprimaient les récepteurs du CD40L : CD40, $\alpha_{IIb}\beta_3$ et $\alpha_5\beta_1$ et libéraient du sCD40L. Le sCD40L seul a induit une activation de NF-κB plaquettaire. Cet effet était absent des plaquettes de souris déficientes en CD40 ($CD40^{-/-}$) et inhibé par le blocage de CD40, mais n'était pas affecté par le blocage de $\alpha_{IIb}\beta_3$ ou $\alpha_5\beta_1$. L'axe sCD40L/CD40 a aussi activé TAK1 en amont de NF-κB. Dans les études fonctionnelles, le sCD40L seul n'a pas eu d'effet sur l'agrégation plaquettaire, mais a potentialisé l'agrégation en présence de doses sous-optimales de thrombine; cet effet a été aboli

par des inhibiteurs de CD40, TAK1 et NF-κB. Cette première étude nous a permis de conclure que le sCD40L amorce les plaquettes *via* des voies de signalisation impliquant CD40/TAK1/NF-κB, ce qui prédispose les plaquettes à une activation et agrégation accrues en réponse à des stimuli thrombotiques.

Dans le deuxième volet de mon projet, nous avons émis l'hypothèse qu'en présence de taux élevés de sCD40L, l'efficacité de l'ASA peut varier et avons visé à déterminer les effets de l'ASA sur la signalisation et l'agrégation des plaquettes en présence de sCD40L. Les effets de l'ASA sur les plaquettes humaines traitées au sCD40L, en réponse à des concentrations sous-optimales de collagène ou de thrombine, ont été évalués sur l'agrégation, la sécrétion de thromboxane A_2 (TxA_2) et la phosphorylation de p38 MAPK, NF-κB, TAK1 et la chaîne légère de la myosine (MLC). Le sCD40L a significativement augmenté la sécrétion de TxA_2 par les plaquettes, en réponse à des doses sous-optimales de collagène et de thrombine; cet effet a été inversé par l'ASA. L'ASA n'a pas inhibé la phosphorylation de p38 MAPK, NF-κB, TAK1, que ce soit avec une stimulation plaquettaire par le sCD40L seul ou en présence des agonistes. Le sCD40L a potentialisé l'agrégation plaquettaire, un effet complètement inversé et partiellement réduit par l'ASA en réponse à une dose sous-optimale de collagène et de thrombine, respectivement. Les effets de l'ASA sur les plaquettes traitées au sCD40L avec du collagène étaient liés à l'inhibition du changement de forme des plaquettes et à la phosphorylation de la MLC. En résumé, l'ASA n'affecte pas la signalisation plaquettaire du sCD40L, mais empêche son effet sur la sécrétion de TxA_2 et l'agrégation plaquettaire en réponse au collagène, *via* un mécanisme impliquant l'inhibition de la MLC.

En conclusion, ce projet nous a permis de déterminer que l'amorçage des plaquettes par le CD40L *via* l'activation de NF-κB dépend du récepteur CD40 et la signalisation *via* TAK1. Ainsi, l'axe sCD40L/CD40/TAK1/NF-κB potentialise l'activation et l'agrégation en réponse à des stimuli thrombotiques, ce qui peut favoriser l'occurrence d'événements athéro-thrombotiques chez les patients coronariens. De plus, étant donné que l'ASA n'a pas d'effet sur la signalisation *via* l'axe sCD40L, le ciblage de cet axe dans les plaquettes peut avoir un potentiel thérapeutique chez les patients coronariens présentant des taux élevés de sCD40L et ne réagissant pas ou moins à l'ASA.

Mots-clés : CD40L/CD40, NF-κB, Aspirine, Plaquette, Thrombose

Abstract

CD40 ligand (CD40L) is a thrombo-inflammatory molecule that predicts cardiovascular events. Platelets are the main source of soluble CD40L (sCD40L) in the circulation, with the ability to influence platelet function through its binding to platelet receptors: CD40, $\alpha_{IIb}\beta_3$, and $\alpha_5\beta_1$. We have previously demonstrated that stimulation of human platelets with sCD40L induces activation of nuclear factor kappa B (NF-κB), which may play a non-genomic role in priming platelets. Indeed, platelet priming by sCD40L strongly enhances platelet activation and aggregation in response to suboptimal doses of agonists. However, the involvement of the different receptors in platelet priming following activation of NF-κB by sCD40L remains unknown. In addition, the transforming growth factor-B (TGF-B) -activated Kinase (TAK1), a major regulator of inflammation, is activated by sCD40L in nucleated cells, and may be involved in sCD40L/NF-κB signaling. On the other hand, sCD40L is high in the bloodstream of coronary patients, who receive Aspirin (ASA) for secondary prevention. However, antiplatelet therapy with ASA is ineffective in some patients and its efficacy is reduced in patients at high risk for coronary events, increasing their risk of platelet over-activation and developing thrombosis. High blood levels of thrombo-inflammatory mediators, such as sCD40L, may explain such variability. Thus, we undertake this project to elucidate the impact of the sCD40L/NF-κB axis on platelet function and its modulation by ASA.

In the first part of our project, we aimed to identify the sCD40L receptors involved in the activation of platelet NF-κB, their downstream signaling and their involvement in platelet aggregation. We have shown that platelets express CD40L receptors: CD40, $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ and release sCD40L. sCD40L alone induced activation of platelet NF-κB. This effect was absent in $CD40^{-/-}$ mouse platelets and inhibited by CD40 blockade, but was not affected by $\alpha_{IIb}\beta_3$ or $\alpha_5\beta_1$ blockade. The sCD40L/CD40 axis has also activated TAK1 upstream of NF-kB. In functional studies, sCD40L alone had no effect on platelet aggregation but potentiated aggregation in the presence of suboptimal thrombin doses. The inhibitors of CD40, TAK1 and NF-κB abolished this effect. This first study concluded that sCD40L primes platelets via CD40/TAK1/NF-κB signaling pathways, which predisposes platelets to increased activation and aggregation in response to thrombotic stimuli.

In the second part of our project, we hypothesized that in the presence of high levels of sCD40L; the effectiveness of ASA may vary. Accordingly, we aimed at determining the effects of ASA on signaling and aggregation of platelets in the presence of sCD40L. The effects of ASA on human platelets treated with sCD40L, in response to suboptimal concentrations of collagen or thrombin, were evaluated on aggregation, thromboxane A_2 (TxA_2) secretion and phosphorylation p38 MAPK, NF-κB, TAK1 and myosin light chain (MLC). sCD40L significantly increased TxA_2 secretion by platelets in response to suboptimal doses of collagen and thrombin; this effect has been reversed by the ASA. ASA did not inhibit the phosphorylation of p38 MAPK, NF-κB, TAK-1, with platelet stimulation by sCD40L either alone or in the presence of agonists. sCD40L potentiated platelet aggregation, an effect completely reversed and partially reduced by ASA in response to a suboptimal dose of collagen and thrombin, respectively. In addition, the effects of ASA on sCD40L-treated platelets with collagen related to inhibition of platelet shape change and phosphorylation of MLC. In summary, ASA does not affect platelet signaling *via* sCD40L, but prevents its effect on TXA_2 secretion and platelet aggregation in response to collagen, *via* a mechanism involving inhibition of MLC.

In conclusion, this project allowed us to determine that platelet priming by sCD40L *via* activation of NF-κB depends on the CD40 receptor and signaling via TAK1. Thus, the sCD40L/CD40/TAK1/NF-κB axis potentiates activation and aggregation in response to thrombotic stimuli, which may promote occurrence of atherothrombotic events in coronary patients. In addition, since ASA has no effect on platelet signaling via the sCD40L axis, targeting of this axis in platelets may have therapeutic potential in coronary patients with high levels of sCD40L that are none or less responding to ASA.

Key words: CD40L/CD40, NF-κB, Aspirin, Platelets, Thrombosis

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Liste des abréviations

AA : Acide arachidonique

AC : Adenylate cyclase

ADAM : *A disintegrin and metalloproteinase domain-containing protein*

ADAMTS : *A Disintegrin and metalloproteinase with thrombospondin motifs*

ADP : Adénosine diphosphate

AINS : Anti-inflammatoires non stéroïdiens

AIT : Accident ischémique transitoire

AMPc : Adénosine monophosphate cyclique

ANO6 : Anoctamin-6

ARNm : ARN messager

ASA : Acide salicylique ou aspirine

ATP : Adénosine triphosphate

AVC : Accidents vasculaires cérébrales

Ca^{2+} : Ion calcium

CD : *Cluster of differentiation*

CD40L : *CD40 Ligand*

Cdc42 : *Cell division cycle 42*

CLEC-2 : *C-type lectinlike receptor 2*

CMH : Complexe majeur d'histocompatibilité

CML : Cellules musculaires lisses

COX-1 : Cyclooxygénase-1

CPA : Cellules présentatrices d'antigènes

CYP450 : Cytochrome P450

DAG : Diacylglycérol

DRO : Dérivés réactifs de l'oxygène

ENTPD1 : Ectonucleoside triphosphate diphosphohydrolase 1

EP : Embolie pulmonaire

ERK : *Extracellular signal-regulated protein kinase*

FAK : *Focal adhesion kinase*

FasL : *Fas ligand*

FcRγ : *Fc receptor γ-chain*

FDA : *Food and drug administration*

GC : Guanylate cyclase

GEF : *Guanine nucleotide exchange factor*

GP: Glycoprotéine

GPCR : Récepteurs couplés aux protéines G

GTP : Guanosine triphosphate

HIGM : Hyper-IgM lié à l'X

ICAM-1 : *Intercellular adhesion molecule-1*

ICP : Intervention coronarienne percutanée

IFN : Interferon

IGF-1 : *Insulin-like growth factor-1*

Ig : Immunoglobuline

IL : Interleukine

IM : Infarctus du myocarde

IP3 : Inositol 1,4,5-trisphosphate

ITAM : *Immunoreceptor tyrosine-based activation motif*

K^+ : Ion potassium

kDa : Kilodaltons

KGD : Lysine-Glycine-Acide aspartique

LDL : *Low-density lipoprotein*

Mac-1 : *Macrophage antigen-1*

MAP : Maladie artérielle périphérique

MAPK : *Mitogen-activated protein kinases*

MCP-1 : *Monocyte chemoattractant protein 1*

Mg^{2+} : Ion magnesium

MIDAS : *Metal ion-dependent adhesion site*

MIP-1α : *Macrophage inflammatory protein 1-alpha*

MLC : *Myosin light chain*

MLCK : *Myosin light chaine kinase*

MMP : *Matrix metalloproteinase*

MP : Microparticules plaquettaires

MRP4 : *Multidrug resistance protein 4*

Munc : *Mammalian uncoordinated*

NADPH : Nicotinamide adenine dinucleotide phosphate

NF-κB : *Nuclear factor-κB*

NOS : *Nitric oxide synthase*

OMS : Organisation mondiale de la santé

oxLDL : LDL oxydé

PAC-1 : *Procaspase activating compound*

PAR : *Protease activated receptor*

PDGF : *Platelet derived growth factor*

PF4 : *Platelet factor 4*

PG : Prostaglandine

PI3K : Phosphoinositide 3-kinase

PIP2 : Phosphatidylinositol 4,5-bisphosphate

PKC : Protein kinase C

PLA : Phospholipases A

PLC : Phospholipase C

PSGL-1: *P-selectin glycoprotein ligand-1*

PSI : Plexines, semorphines et intégrines

PtdSer : Phosphatidylsérine

RANK : *Receptor activator of NF-Kb*

RANTES : *Regulated on activation, normal T cell expressed and secreted*

Rap1b : *Ras-related protein 1*

RGD : Arginine-Glycine-Acide aspartique

RhoGEF : *Rho specific guanine nucleotide exchange factors*

RIAM : *Rap1-interacting adaptor molecule*

ROCK : Rho kinase

SCA : Syndrome coronarien aigu

SCO : Système canaliculaire ouvert

SDF-1 : *Stromal cell-derived factor 1*

SLP-76 : *SH2 domain containing leukocyte protein of 76 kDa*

SNAP : *Soluble N-ethylmaleimide sensitive factor-associated protein*

SNARE : *Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor*

Src : Proto-oncogene tyrosine-protein kinase

STD : Système tubulaire dense

Syk : *Spleen tyrosine kinase*

TAP1 : *Antigen peptide transporter-1*

TCR : *T cell receptor*

TF : Facteur tissulaire

TNF : *Tumour necrosis factor*

TP : *Thromboxan/prostaglandin receptor*

TRAF : *TNF receptor associated factor*

TRAIL : *TNF-related apoptosis-inducing ligand*

TSP-1 : Thrombospondin-1

TVP : Thrombose veineuse profonde

TxA_2 : Thromboxane A_2

VAMP : *Vesicules Associated Membrane Proteins*

VASP : *Vasodilator-stimulated phosphoprotein*

VCAM-1 : *Vascular cell adhesion molecule-1*

VEGF : *Vascular endothelial growth factor*

VWF : Facteur Von Willebrand

WT : *Wild type*

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Chapitre 1 : Les plaquettes

1.1 Origine et morphologie

1.1.1 Origine

La première description précise des plaquettes, aussi dénommées thrombocytes, provient des études de Max Schultze en 1865 (1). Selon sa description, il représente les plaquettes en tant que le « troisième élément » dans le sang, et donc indépendantes des érythrocytes et leucocytes, en leur proposant le terme « masses granulaires », enchevêtrées d'un réseau fibreux. En 1881-82, Giulio Bizzozero (2, 3) a approfondi la notion de ce « troisième élément » en leur attribuant le nom « petite plaque ». Il les décrit comme étant des corps discoïdes anucléés, consistant en une membrane et une matrice contenant des granules dispersés. De plus, il assimila à ces « petites plaques » un rôle dans l'hémostase et la thrombose vasculaire puisqu'il remarqua qu'elles ont la capacité d'adhérer à la paroi d'un vaisseau lésé. Enfin, hormis les plaquettes, il est aussi le premier à identifier les mégacaryocytes de la moelle osseuse sans observer un lien entre les mégacaryocytes et les plaquettes (4). En effet, ce lien est suggéré plus tard par Wright (5) qui identifia les mégacaryocytes en étant les cellules précurseurs des plaquettes. En 1886, Osler (6) reconnait la contribution des plaquettes dans les maladies thrombotiques en découvrant des thrombi dans des lésions athéromateuses aortiques.

Les plaquettes proviennent de cellules hautement spécialisées, les mégacaryocytes. Ces cellules se développent à partir de cellules souches hématopoïétiques au niveau de la moelle osseuse grâce à un processus qui se dénomme la mégacaryopoïèse. Ce processus permettant la formation de mégacaryocytes matures est hautement régulé et complexe. Les cellules souches hématopoïétiques se différencient progressivement en des progéniteurs multipotents, suivis par des progéniteurs mégacaryocytaires-érythroïdiens, dont le devenir de ces cellules peut être soit les érythrocytes ou mégacaryocytes. Finalement, avant que les mégacaryocytes soient capables de produire des plaquettes, elles subissent une panoplie de changements phénotypiques où on assiste à une augmentation de leurs tailles, un mécanisme de multiplication vers une polyploïdisation, un processus appelé l'endomitose, et la formation d'un système de membrane de démarcation, d'invagination de la membrane cytoplasmique et des granules. Ces myriades de changements phénotypiques permettent enfin aux mégacaryocytes de produire environ 100 milliards de plaquettes fonctionnelles par jour (7).

1.1.2 Morphologie

Les plaquettes sont de forme discoïde au repos; or lors de leurs activations, elles adoptent une forme généralement sphérique avec une formation d'extensions membranaires appelées filopodes, ou encore, au contact d'une surface, ces extensions peuvent se développer en des feuillets membranaires appelés lamellipodes. Les plaquettes sont dépourvues d'un noyau et sont les composantes les plus minimes du système sanguin, avec une dimension moyenne de 3 x 0.5 µm. Leurs contenus cytoplasmiques, tels que les mitochondries, les lysosomes et les membranes résiduelles du réticulum endoplasmique proviennent des mégacaryocytes. Les plaquettes circulent à une concentration de 150 à 450 x 10^6 plaquettes par ml chez l'adulte avec une durée de vie moyenne de 10 jours. Leur élimination a lieu au niveau de la rate, le foie et le système réticulo-endothélial (8). La morphologie des plaquettes peut être divisée en trois structures : la membrane plasmique, le cytosquelette et le cytoplasme (Figure 1.1).

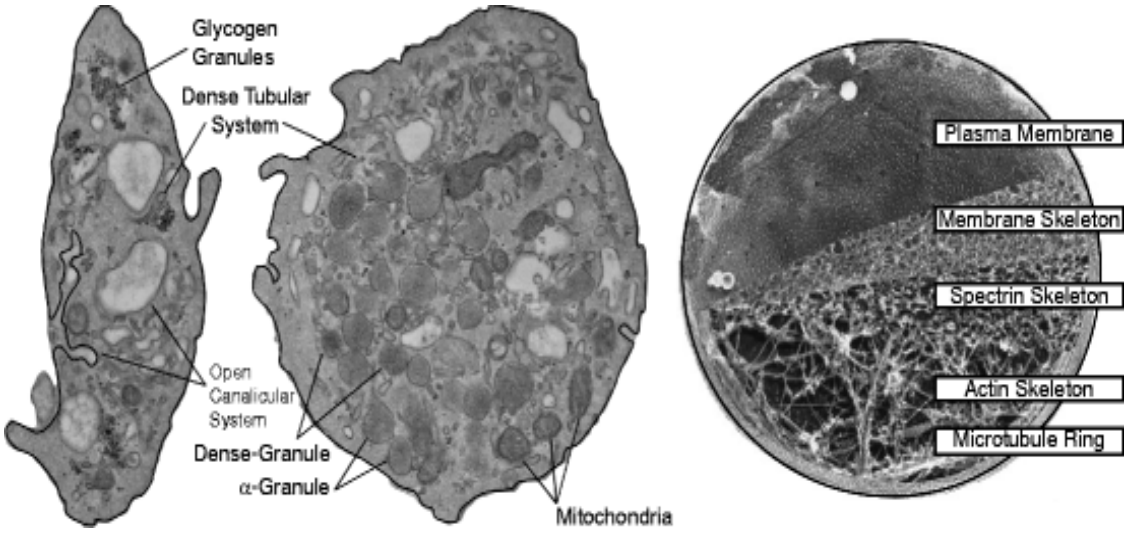

Figure 1.1: Structure des plaquettes observées sur des micrographies électroniques à coupes minces de plaquettes discoïdes. Hartwig JH et al. *Thromb Haemost.* 1999 Aug;82(2):392-8.

1.1.2.1 La membrane plasmique

La membrane plasmique est composée d'une bicouche de phospholipides constituée de cholestérol libre et de lipide neutre; cela favorise le maintien de la stabilité des membranes. Au niveau de la membrane sont ancrés les récepteurs plaquettaires essentiels à la fonction

plaquettaire tels que les glycoprotéines qui représentent la majorité des protéines sur la membrane (9).

La membrane plasmique comporte de nombreuses invaginations membranaires qui créent le système canaliculaire ouvert (SCO). Ce système représente un conduit d'entrée d'éléments externes dans les plaquettes et la relâche du contenu des granules de sécrétions dans le milieu extracellulaire suite à l'activation plaquettaire. Ce système est aussi une importante réserve de membrane mobilisable permettant la formation des extensions membranaires, dont les filopodes. Au niveau de la membrane, on discerne le système tubulaire dense (STD), correspondant au réseau réticulum endoplasmique lisse qui se trouve dans les mégacaryocytes et les enzymes responsables de la synthèse des prostaglandines. Il semble avoir une connexion entre les deux systèmes membranaires (SCO et STD) pour permettre le relâche du contenu des granules de sécrétions (9, 10).

1.1.2.2 Le cytosquelette

Le cytosquelette est composé d'une part d'une monocouche de filaments microtubulaires emmaillotés sous la membrane plasmique responsable du maintien de la forme discoïde des plaquettes au repos. D'autre part, on distingue une couche à base de spectrine et de filaments d'actine favorisant la liaison du réseau d'actine avec différentes glycoprotéines membranaires (11, 12). De plus, ce réseau offre une résistance aux forces de cisaillement engendrées par le débit sanguin en maintenant l'intégrité plaquettaire. Le changement de forme plaquettaire lors de l'activation plaquettaire est le résultat du dynamisme de désassemblage et d'assemblage des microtubules et d'actine du cytosquelette entrainant la transition des plaquettes d'une forme discoïde à une forme sphérique avec émission de filopodes (13).

1.1.2.3 Le cytoplasme

Le cytoplasme des plaquettes contient plusieurs types d'organites, dont les mitochondries, les granules de glycogènes et les granules de sécrétions. Il y a trois types de granules : granules α, granules denses et lysosomes. Cependant, les granules α et dense sont les principales réserves plaquettaires contenant une panoplie de protéines cruciales au fonctionnement plaquettaire (voir **section 1.3.3**). Les granules sont placés à proximité du SCO. Dès lors, suite à l'activation plaquettaire, elles fusionnent et relâchent leur contenu dans le milieu extracellulaire (9).

1.2 Les récepteurs plaquettaires

Aux sites de lésion vasculaire, les interactions entre les récepteurs et les ligands plaquettaires sont cruciales à l'efficacité du recrutement de plaquettes circulant sur les composantes de la matrice sous-endothéliale exposée ainsi que pour l'activation plaquettaire ultérieure et leur agrégation. Au niveau des vaisseaux de petits diamètres où les forces de cisaillement sont élevées, l'interaction entre le complexe glycoprotéique Ib-IX-V (GPIb-IX-V) et le facteur Von Willebrand (VWF) est primordiale pour le recrutement des plaquettes sur la lésion vasculaire (14). Suite au recrutement des plaquettes, elles adhèrent et s'activent grâce à plusieurs familles de récepteurs plaquettaires telles que les intégrines ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ et $\alpha_{IIb}\beta_3$) et les immunorécepteurs à motifs ITAM comme la GPVI- FcRγ et le CLEC-2 (15, 16). Suite à cela, la signalisation «*inside-out*» de l'intégrine $\alpha_{IIb}\beta_3$, en réponse à l'activation plaquettaire mène à l'agrégation plaquettaire grâce à la liaison du $\alpha_{IIb}\beta_3$ au fibrinogène, un phénomène amplifié par des récepteurs couplés aux protéines G dont les récepteurs purinergiques ($P2Y_1$ et $P2Y_{12}$), les récepteurs activés par les protéases (PARs) et les récepteurs de thromboxane (TP). Notamment, d'autres récepteurs s'impliquent aussi dans la fonction plaquettaire, mais on discutera dans cette section des récepteurs les plus pertinents.

1.2.1 Le GPIb-IX-V, le récepteur de recrutement plaquettaire

Suite à une lésion vasculaire, les fibrilles de collagène sous-endothéliales sont exposées à la circulation ainsi que d'autres facteurs sous-endothéliaux tels que le VWF, la fibronectine, la laminine, l'ostéopontine, la vitronectine, etc. Malgré que le VWF sous-endothélial soit déjà lié au collagène VI (17), les fibrilles de collagène I et III recrutent davantage de VWF circulants, cela favorise la formation d'un grand réseau multimérique de VWF, entrainant le recrutement et le roulement initial des plaquettes aux sites de liaison grâce au récepteur GPIb-IX-V (18).

Le complexe GPIb-IX-V est composé de quatre sous-unités de protéines transmembranaires, GPIbα, GPIbβ, GPIX, et GPV. Elles sont assemblées en un complexe fonctionnel grâce à la molécule chaperonne gp96 dans le réticulum endoplasmique des mégacaryocytes à la surface de la moelle osseuse (19). Le GPIb-IX-V est le deuxième plus abondant récepteur sur la membrane plaquettaire avec 25 000 copies par plaquettes de GPIb-IX et 12 500 copies de GPV(20). Il est omniprésent lors de la fonction plaquettaire puisqu'il est impliqué dans l'hémostase primaire et secondaire. En effet, hormis son rôle dans le recrutement initial des

plaquettes sur la lésion vasculaire par sa liaison au VWF, le GPIb-IX-V permet l'adhérence plaquettaire par sa liaison à la P-séléctine de l'endothélium (21). Il est aussi impliqué dans le recrutement des leucocytes sur l'endothélium par sa liaison au Mac-1 leucocytaire (22, 23). De plus, le GPIb-IX-V peut se lier à la thrombine à de faibles concentrations (24), mais son implication dans l'activation plaquettaire est un sujet de débat. Une théorie propose que le GPIb-IX-V ne peut pas initier une réponse plaquettaire tout seul, mais joue plutôt le rôle d'un quai pour la thrombine afin de faciliter le clivage des récepteurs à la thrombine, les PARs (25). Cependant, une autre théorie suggère que le GPIb-IX-V est capable d'induire une réponse en liant la thrombine par sa forme inactive et donc d'agir en tant qu'un récepteur de thrombine indépendamment des PARs (26, 27). Enfin, une étude ne supportant ni l'une ni l'autre, démontre que la thrombine induit une signalisation spécifique au GPIb-IX-V qui est dépendante de la signalisation des PARs afin d'enclencher une activation plaquettaire (28). Enfin, le GPIb-IX-V est impliqué dans la coagulation puisqu'il est un récepteur des facteurs de coagulation XII et XI (29, 30). Au niveau intracellulaire, le GPIb-IX-V interagit avec la filamine A permettant sa liaison avec le cytosquelette d'actine favorisant son ancrage membranaire. De plus, d'autres partenaires s'associent aussi de manière constitutive au complexe GPIb-IX-V, soit la calmoduline (31) et la protéine adaptatrice 14-3-3ζ qui sont aussi liées à la sous-unité p85 du PI3K (32). Ces interactions jouent un rôle dans l'adhésion et le changement de forme plaquettaire dépendante du complexe GPIb-IX-V et interviennent dans la signalisation transmembranaire (31, 33, 34). Enfin, l'activation du GPIb-IX-V par le VWF induit l'association du PI3K à la tyrosine kinases de la famille Src qui phosphoryle Syk; ce dernier active la phospholipase C γ2 (PLCγ2) qui mène à une signalisation impliquant le phosphoinositide 3-kinase (PI3K) et les protéines kinases C (PKC). Cette signalisation permet le changement de forme plaquettaire qui consiste en leur contraction et émission de filopodes ainsi qu'à l'activation de l'intégrine $\alpha_{IIb}\beta_3$, une étape essentielle pour l'initiation de l'activation plaquettaire (35) (Figure 1.2). Ainsi, l'absence du complexe GPIb-IX-V suite à une mutation des trois gènes codant le GPIbα, GPIbβ et le GPIX engendre le syndrome de Bernard-Soulier qui se caractérise par des syndromes hémorragiques sévères chez l'individu (36).

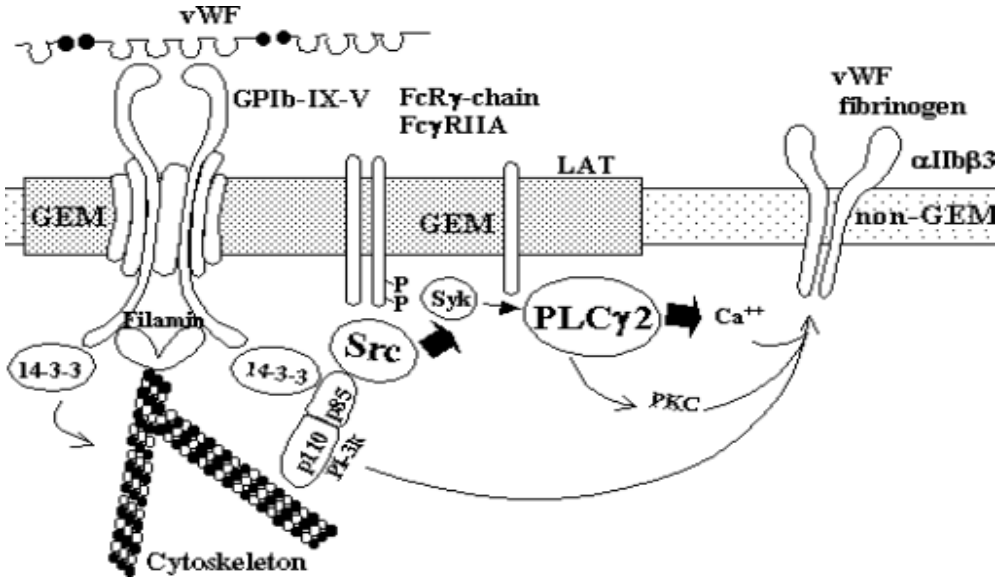

Figure 1.2: La voie de signalisation du complexe GPIb-IX-V. Ozaki. Y et al. J Thromb Haemost. 2005 Aug; 3(8):1745-51.

1.2.2 Les récepteurs d'adhésion et d'activation

1.2.2.1 GPVI-FcRγ

La GPVI appartient à la superfamille des immunoglobulines et elle est composée de deux domaines C2 similaires à ceux des immunoglobulines. Au niveau de ces domaines, fortement glycosylés, se trouve le site de reconnaissance spécifique à la forme en triple hélice du collagène. En effet, la GPVI et l'intégrine $\alpha_2\beta_1$ constituent les récepteurs plaquettaires principaux du collagène (37). La caractérisation du GPVI a eu lieu grâce à des études effectuées auprès de patients dont les plaquettes ne répondent pas à des stimulations au collagène. Grâce à des études de radio-marquages, les plaquettes de ces patients indiquent une absence d'une protéine d'environ 61 kDa qui est identifiée comme étant la GPVI (38). La GPVI est uniquement exprimée sur les plaquettes et les mégacaryocytes (3 700 copies par plaquettes). Elle s'associe avec l'adapteur transmembranaire FcRγ par son domaine C-terminal et un résidu d'arginine de la région transmembranaire pour former un complexe GPVI-FcRγ. En effet, l'expression de la GPVI à la membrane dépend de la stabilisation du complexe grâce au résidu d'arginine de la GPVI qui forme un pont salin avec un acide aspartique du FcRγ (39). Ce complexe est indispensable pour le processus de signalisation plaquettaire; en effet, une fois la GPVI activée

par le collagène, les motifs ITAM (*Immunoreceptor tyrosine-based activatory motifs*) se trouvant au niveau des chaines de FcRγ sont phosphorylés par la famille des tyrosines kinases Src, menant au recrutement et l'activation de la kinase Syk (40). Suite à cela, une cascade de signalisation incluant l'activation PLCγ2 et des isoformes de PKC, les PI3K et le SLP-76 (*SH2 domain containing leukocyte protein of 76 kDa*) permet l'adhésion et l'activation des plaquettes (41). Suite à l'activation plaquettaire, la métalloprotéinase ADAM10/17 clive la GPVI; cette action peut représenter un mécanisme de régulation négative de l'activité plaquettaire (42) (Figure 1.3). La GPVI possède une affinité faible pour le collagène et permet l'adhésion des plaquettes en condition statique (43, 44). Cependant, en présence d'un flux sanguin élevé, la GPVI contribue à l'activation des intégrines telles que $\alpha_2\beta_1$ et $\alpha_{IIb}\beta_{III}$ grâce à la sécrétion d'ADP et TxA_2 qui mène à une adhésion stable des plaquettes ainsi que leur étalement et leur agrégation (45, 46).

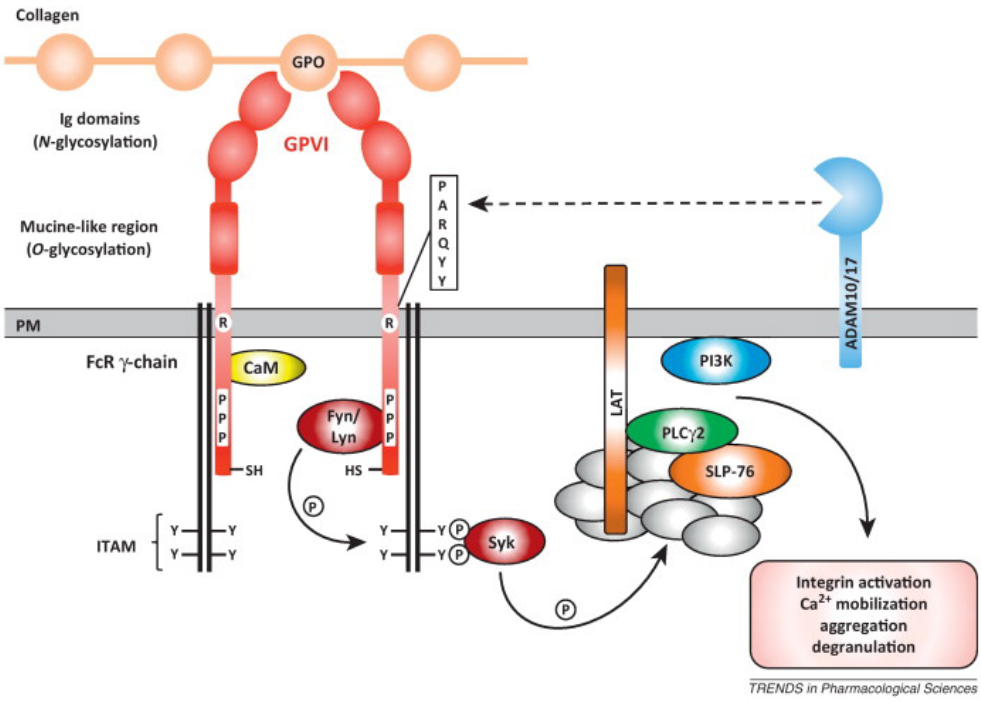

Figure 1.3: Structure et signalisation du complexe GPVI-FcRγ. Dutting. S et al. *Trends Pharmacol Sci.* 2012 Nov; 33(11):583-90

1.2.2.2 $\alpha_2\beta_1$

L'intégrine $\alpha_2\beta_1$ (GPIa/IIa) est un hétéro-dimère composé de deux sous-unités, une chaine α_2 et une chaine β_1; son niveau d'expression est d'environ 4000 copies par plaquettes. La sous-unité α_2 contient, dans sa partie extracellulaire, le domaine I indispensable pour la liaison du collagène (47). Cette liaison du domaine I de la sous-unité α_2 au collagène est dépendante d'un site de reconnaissance pour des ions magnésium (Mg^{2+}) dénommé MIDAS (*metal ion-dependent adhesion site*) (48). En effet, l'intégrine $\alpha_2\beta_1$ est le premier récepteur de collagène à être caractérisée sur les plaquettes (49). Elle a pour fonction principale l'adhésion stable des plaquettes aux fibres de collagène exposées au niveau d'une lésion vasculaire (46). L'activation de $\alpha_2\beta_1$ par des agonistes plaquettaires, comme on l'a mentionné par le biais de la GPVI ou les agonistes solubles contenus dans les granules (TxA_2, ADP et autres), induit une signalisation «*inside-out*» qui est un mécanisme d'activation commun au niveau des intégrines telle que l'intégrine primordiale à la fonction plaquettaire, l'$\alpha_{IIb}\beta_3$ (50) (Figure 1.5). Cette signalisation met en jeu une cascade impliquant les PLCγ2 qui hydrolysent le phosphatidylinositol 4,5-bisphosphate (PIP2) de la membrane plasmique en diacylglycérol (DAG) et en inositol 1,4,5-trisphosphate (IP3) et la mobilisation du calcium (Ca^{2+}) contenu dans le STD (51). La génération de DAG et la mobilisation du Ca^{2+} induisent aussi l'activation des isoformes de PKC et les PI3K ainsi qu'une protéine GEF (*Guanine nucleotide exchange factor*) qui se dénomme CalDAG-GEFI (52). L'activation de ces effecteurs engendre l'activation du Rap1b qui s'associe à des protéines du cytosquelette dont RIAM (*Rap1-interacting adaptor molecule*), taline et kindline-3 et avec les sous-unités intracellulaires de l'intégrine β_1 (ou β_3 comme dans le cas l'$\alpha_{IIb}\beta_3$). Cette association mène finalement au changement conformationnel permettant l'activation *«inside out»* des intégrines (53-56). De plus, un peptide spécifique pour l'$\alpha_2\beta_1$, GFOGER, révèle que cette intégrine peut notamment déclencher une signalisation *«outside-in»*, et similairement au GPVI, elle fait intervenir la cascade de signalisation impliquant les tyrosines kinases Src et l'activation de la kinase Syk, et PLCγ2. Cette signalisation induit le changement de forme des plaquettes avec émission de filopodes et son étalement *via* la formation des lamellipodes (57). Enfin, malgré qu'elle joue un rôle important dans l'adhésion plaquettaire, la GPVI s'avère être le récepteur le plus essentiel à la réponse des plaquettes au collagène. En effet, l'affinité de l'intégrine $\alpha_2\beta_1$ au collagène dépend de l'activation plaquettaire. De plus, une déficience en α_2 ou β_1 chez la souris démontre qu'il y a peu d'effet sur la capacité des plaquettes à adhérer au collagène et de s'activer; elle joue donc un rôle de support de l'adhésion plaquettaire (58, 59).

Cela peut s'expliquer par la liaison de $\alpha_{IIb}\beta_3$ au VWF suite à l'activation plaquettaire par la GPVI. Cependant, des polymorphismes de $\alpha_2\beta_1$ entrainant sa surexpression membranaire pourraient entrainer des facteurs de risque qui favorisent l'occurrence de pathologies thrombotiques (60-62).

1.2.2.3 $\alpha_5\beta_1$

L'intégrine $\alpha_5\beta_1$ est le récepteur plaquettaire principal de la fibronectine qu'elle lie *via* la séquence RGD (Arginine-Glycine-Acide aspartique) (63). Ce récepteur peut adhérer à la fibronectine dans des conditions statiques n'impliquant pas l'activation plaquettaire (64). Cette affinité augmente suite à l'activation «*inside-out*» de l'intégrine $\alpha_5\beta_1$ similaire à celle des intégrines comme mentionné dans la section ci-dessus (65). Cependant, elle est incapable d'engendrer une activation des tyrosines kinases et donc le changement de forme plaquettaire; elle est ainsi dépendante de l'activation de l'intégrine $\alpha_{IIb}\beta_3$ (64). De même, dans des conditions de flux sanguin à cisaillement élevé, $\alpha_5\beta_1$ est responsable partiellement de l'adhésion plaquettaire à la fibronectine, mais dépendent de l'activation de l'$\alpha_{IIb}\beta_3$ aussi (66). Ainsi, l'intégrine $\alpha_5\beta_1$ a un rôle limité à l'initiation de l'adhésion des plaquettes en repos à la fibronectine ou dans de grands vaisseaux sanguins où les forces de cisaillement sont basses. Cette adhésion est davantage plus importante suite à l'implication d'autre intégrines comme l'$\alpha_{IIb}\beta_3$.

1.2.2.4 $\alpha_6\beta_1$

L'intégrine $\alpha_6\beta_1$ est le récepteur plaquettaire principale de la laminine, et similairement à $\alpha_5\beta_1$, ne requiert pas que les plaquettes soient activées pour lier son ligand, la laminine (67). L'adhésion de l'intégrine $\alpha_6\beta_1$ à la laminine entraine une signalisation *«outside-in»* impliquant surtout l'activation du PI3K et Cdc42 (*cell division cycle 42*) et moins les tyrosines kinases Src. Cette activation permet surtout la formation de filopodes permettant le changement de forme des plaquettes (68). Cependant, la stabilisation de la liaison de la laminine à l'intégrine $\alpha_6\beta_1$ permet à la GPVI, qui a une affinité faible à la laminine, de lier ce dernier; cela permet l'étalement des plaquettes suite à la formation de lamellipodes (69).

1.2.2.5 $\alpha_{IIb}\beta_3$

L'intégrine $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) est le récepteur plaquettaire le plus abondant sur la surface plaquettaire avec une expression de 80,000 copies par plaquette et avec des réserves supplémentaires (environ 30 000) contenues dans les granules α et le SCO qui peuvent être recrutées à la membrane suite à l'activation plaquettaire. La sous-unité α_{IIb} est composée de deux chaines peptidiques, une chaine lourde contenant une hélice β à sept tours et quatre sites de liaison extracellulaires pour le Ca^{2+} et une chaine légère composée d'un domaine transmembranaire et une région cytoplasmique. Ces deux chaines sont reliées par un pont disulfure. La sous-unité β_3 est par contre constituée d'une région cytoplasmique et trois principaux domaines extracellulaires : un domaine PSI (plexines, semorphines et intégrines) qui contribue à l'activation de l'$\alpha_{IIb}\beta_3$ *via* sa fonction thiol isomérase (70); un domaine I comprenant deux à trois sites de Ca^{2+}, un motif MIDAS impliqué dans la liaison des ligands et deux sites de reconnaissance RGD qui permettent la liaison de différentes protéines comme le fibrinogène, la fibrine, le VWF, la fibronectine et la vitronectine; et un domaine EGF apparenté composé de quatre boucles riches en cystéines qui est impliqué dans le changement de forme de l'intégrine $\alpha_{IIb}\beta_3$ suite à son activation (Figure 1.4) (71).

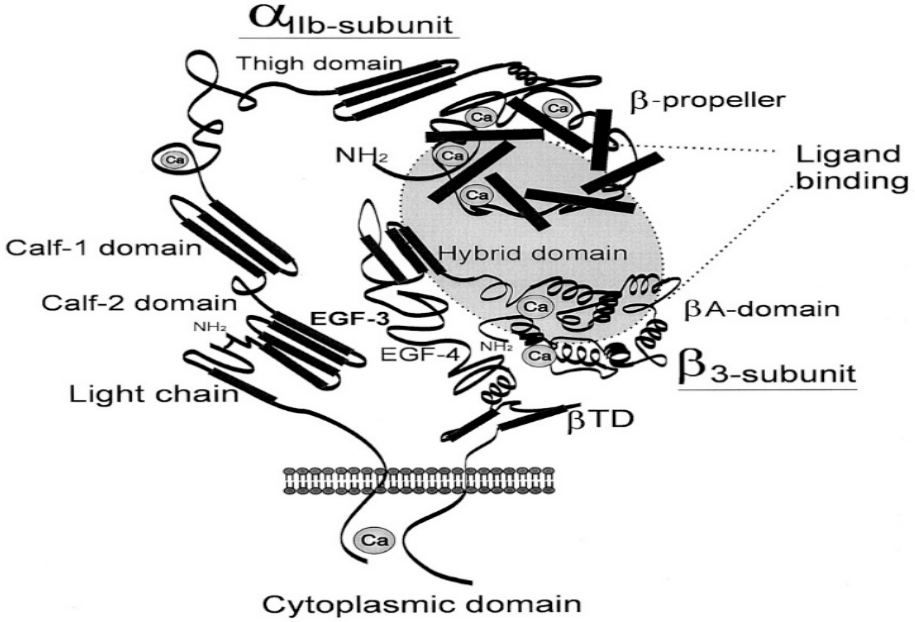

Figure 1.4: Structure de l'intégrine $\alpha_{IIb}\beta_3$. Quinn. MJ et al. *Arterioscler Thromb Vasc Biol.* 2003 Jun 1; 23(6):945-52

En condition de flux sanguin faible, l'intégrine $\alpha_{IIb}\beta_3$ assiste à l'adhésion des plaquettes au site de lésion en s'attachant au fibrinogène exposé et elle lie également le VWF attaché au collagène afin de stabiliser l'adhésion des plaquettes. Cette interaction de l'intégrine avec les protéines exposées au niveau de la lésion induit une signalisation «*inside-out*» (Figure 1.5) permettant de lier les fibrinogènes solubles avec une plus haute affinité (72, 73).

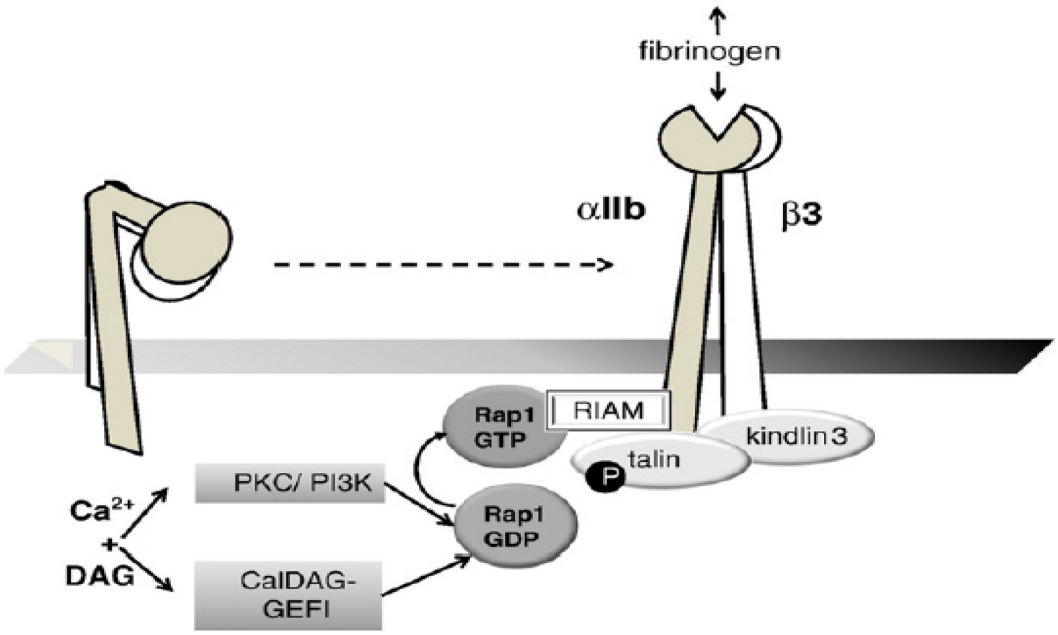

Figure 1.5: Signalisation «*inside-out*» de l'intégrine $\alpha_{IIb}\beta_3$. Broos. K et al. *Blood Rev.* 2011 Jul; 25 (4):155-6725

La nature dimérique du fibrinogène soluble permet le pontage entre deux plaquettes, une étape essentielle à l'agrégation plaquettaire. Suite à cela, la liaison des plaquettes au fibrinogène soluble induit une signalisation *«outside-in»* qui implique des cascades de signalisation de la famille Src qui se trouve au domaine intracellulaire de la chaine β_3 (74). Ces signalisations induisant la phosphorylation de multiples protéines comme les PI3K, les isoformes de PKC, FAK (*focal adhesion kinase)* et les GTPases de la famille Rho consolident l'activation de l'intégrine $\alpha_{IIb}\beta_3$ menant au relargage des granules et le réarrangement cytosquelettique conduisant à l'étalement des plaquettes et la rétraction du caillot (75-78). Ainsi, cette signalisation est primordiale pour la stabilisation des agrégats, un processus fondamental en thrombose et hémostase. En effet, l'absence ou l'altération de l'$\alpha_{IIb}\beta_3$ conduit au syndrome de

thrombasthénie de Glanzmann, une maladie hémorragique caractérisée par un défaut d'agrégation plaquettaire menant à un manque de formation d'un clou hémostatique (79).

1.2.2.6 $\alpha_v\beta_3$

Cette intégrine possède une conformation similaire à l'intégrine $\alpha_{IIb}\beta_3$ et présente ainsi des domaines RGD capables de reconnaitre le VWF, la fibronectine, le fibrinogène et la vitronectine. Cependant, la vitronectine est le ligand privilégié de l'intégrine $\alpha_v\beta_3$. Suite à l'activation plaquettaire, l'$\alpha_v\beta_3$ contribue à l'adhésion des plaquettes sur une surface de fibronectine (64) ou de vitronectine (80). En revanche, son niveau expression est très faible, d'environ 100 à 500 copies par plaquette, suggérant un rôle négligeable dans la fonction plaquettaire (81).

1.2.2.7 CLEC-2

Le CLEC-2 (*C-type lectin-like receptor 2*) fait partie des récepteurs hemi-ITAM, qui se caractérise par une seule séquence TyrxxLeu (YxxL) et il est fortement exprimé sur les plaquettes (2000 copies par plaquettes) (82). Son activation par son ligand physiologique, la podoplanine, induit la phosphorylation du domaine ITAM et l'activation de tyrosine kinase Syk de PLCγ2 et SLP-76, similaire à la signalisation de GPVI, suggérant alors une implication dans l'activation plaquettaire (83-86).

De plus, une dysfonction du récepteur CLEC-2 inhibe l'agrégation plaquettaire et une formation de thrombus défectueuse dans un modèle de thrombose induit par le chlorure de fer chez les souris (87). Curieusement, une étude a démontré que les plaquettes de souris transgénique où le résidu de tyrosine du domaine hemi-ITAM est remplacé par l'alanine retiennent leur capacité d'agréger suite à des stimuli thrombotiques et leur capacité à former des thrombus occlusifs (88). Cela suggère que la phosphorylation de la tyrosine du domaine hemi-ITAM n'est pas requise pour l'activation plaquettaire et que d'autres mécanismes indépendants du domaine hémi-ITAM restent à découvrir (89).

1.2.3 Les récepteurs d'amplification

Les agonistes plaquettaires solubles constituent une source importante dans l'activation plaquettaire et la formation du thrombus. Une fois activées, les plaquettes synthétisent le TxA_2 *via* la cyclooxygénase-1 (COX-1) et relâchent l'adénosine diphosphate (ADP) et l'adénosine

triphosphate (ATP) grâce aux granules denses afin d'amplifier l'activation et l'agrégation plaquettaire en recrutant davantage des plaquettes circulantes afin de former le thrombus (15, 90, 91). Ainsi, parallèlement, l'expression du facteur tissulaire (TF), au niveau de la matrice sous-endothéliale ainsi que sur les leucocytes et les plaquettes activés contribuent à la formation de la thrombine à travers la cascade de la coagulation (92). La thrombine, étant l'agoniste le plus puissant des plaquettes et aussi l'activateur clé de la voie finale de la cascade de la coagulation en convertissant le fibrinogène en fibrine, renforce l'activation et l'agrégation plaquettaire et consolide le thrombus (93).

Les agonistes amplificateurs activent les plaquettes en interagissant avec des récepteurs couplés aux protéines G (GPCR) qui appartiennent à la famille des récepteurs à sept domaines transmembranaires et induisent leurs signaux grâce à des protéines G hétérotrimériques et les canaux ioniques (94). Ainsi, le TxA_2, une fois relâché par les plaquettes activées, amplifie l'activation plaquettaire en liant les récepteurs de thromboxane/prostaglandine (TP). Or, l'ADP lie à son tour deux récepteurs purinérgiques couplés aux protéines G, soit le $P2Y_1$ et le $P2Y_{12}$. Or, l'ATP engendre une activation *via* l'influx calcique en interagissant avec le $P2X_1$ qui est en revanche un canal ionique. Enfin, la thrombine qui se lie, comme on l'a mentionné auparavant, au complexe GPIb-IX-V (voir **section 1.2.1**), interagit par la suite avec les PARs, soit les PAR1 et PAR4 sur les plaquettes humaines et les PAR3 et PAR4 sur les plaquettes de souris (15, 90, 91).

1.2.3.1 TP

L'activation plaquettaire entraine la libération de l'acide arachidonique (AA) à partir des phospholipides membranaires *via* les phospholipases A2 (PLA2). Suite à une conversion enzymatique séquentielle, l'AA génère la prostaglandine G_2 (PGG_2) puis la prostaglandine H_2 (PGH_2) *via* le COX-1, ensuite le PGH_2 est converti en TxA_2 grâce à la thromboxane synthétase. Enfin, le TxA_2 diffuse à l'extérieur de la membrane plaquettaire et amplifie l'activation par leur liaison au récepteur TP (95). Il existe deux isoformes des récepteurs TP, soit, TPα et TPβ; cependant, le TPα domine sur les plaquettes avec environ 1500 copies par plaquette (96). Suite à l'activation par le TxA_2 du TPα qui est couplé aux protéines G_q et $G_{12/13}$, il y a d'une part, une mobilisation du Ca^{2+} intracellulaire et l'activation des isoformes de PKC *via* l'activation du PLCβ par le G_q. D'autre part, il y a une activation de la protéine RhoGEF (*Rho specific guanine nucleotide exchange factors*) *via* $G_{12/13}$ qui mène à la phosphorylation du MLC (*Myosin light*

chain) grâce à l'activation de la protéine RhoA et la Rho Kinase (ROCK)(97-100) (Figure 1.6). Cette signalisation aboutit au changement de forme et sécrétion plaquettaire ainsi que l'agrégation plaquettaire irréversible (98). Enfin, le TxA_2 est une molécule instable avec une demi-vie de 20 à 30 secondes; ainsi, suite à sa courte durée d'action, il est hydrolysé en un métabolite stable et inactif en TxB_2 au niveau de la circulation sanguine (101).

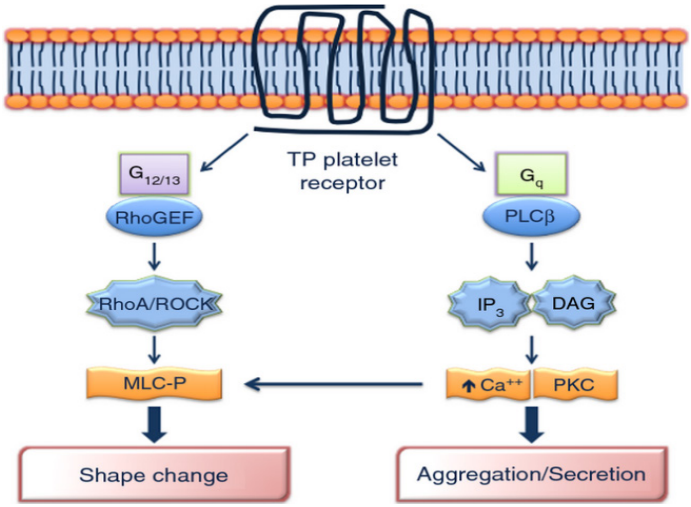

Figure 1.6: Représentation de la voie de signalisation du récepteur TP du TxA_2. Capra. V et al. *J Thromb Haemost*. 2014 Feb;12(2):126-37

1.2.3.2 $P2Y_1$

Le $P2Y_1$ appartient à la famille des récepteurs purinérgiques couplés à la protéine G_q et il est exprimé en environ 150 copies par plaquette. Ainsi, il joue un rôle clé dans l'initiation de la réponse à l'ADP (102). En effet, l'inhibition pharmacologique ou une mutation génétique mène à une absence d'agrégation plaquettaire ou de changement de forme en réponse à l'ADP (103-106). De plus, en réponse à l'ADP, la protéine G_q permet l'activation de la PLCβ et la protéine Rho et une augmentation du Ca^{2+} cytosolique (97, 107). Cela permet un changement de forme plaquettaire et entraine une agrégation rapide et transitoire. Le $P2Y_1$ compte pour environ 20 à 30% des sites de liaison totaux de l'ADP sur la surface des plaquettes. Le $P2Y_1$ contribue notamment à la fonction pro-coagulante des plaquettes (108) ainsi que leur activation par le collagène (104).

1.2.3.3 $P2Y_{12}$

Le $P2Y_{12}$ appartient à la famille des récepteurs purinérgiques couplés à la protéine G_i; il est exprimé en environ 425 copies par plaquette et il est responsable de la plupart des effets

potentialisateurs de l'ADP (109). Une fois activée par l'ADP, la protéine G_i active les PI3K et inhibe l'adenylate cyclase (AC) qui bloque la génération de l'adénosine monophosphate cyclique (AMPc) afin d'empêcher la signalisation PKA/VASP (*Vasodilator-stimulated phosphoprotein*), une voie qui maintient les plaquettes inactives. L'activation du PI3K induit la phosphorylation des protéines effectrices comme les PKC, le PKB et Rap1b (*Ras-related protein 1*) (Figure 1.7) (110). Cette signalisation entraine l'activation des intégrines et joue un rôle crucial dans l'amplification de l'agrégation d'un stade réversible à un stade irréversible. De plus, elle contribue à l'exposition des phospholipides procoagulants. L'importance de $P2Y_{12}$ est observée chez les patients déficients en $P2Y_{12}$ qui présentent des syndromes hémorragiques (111).

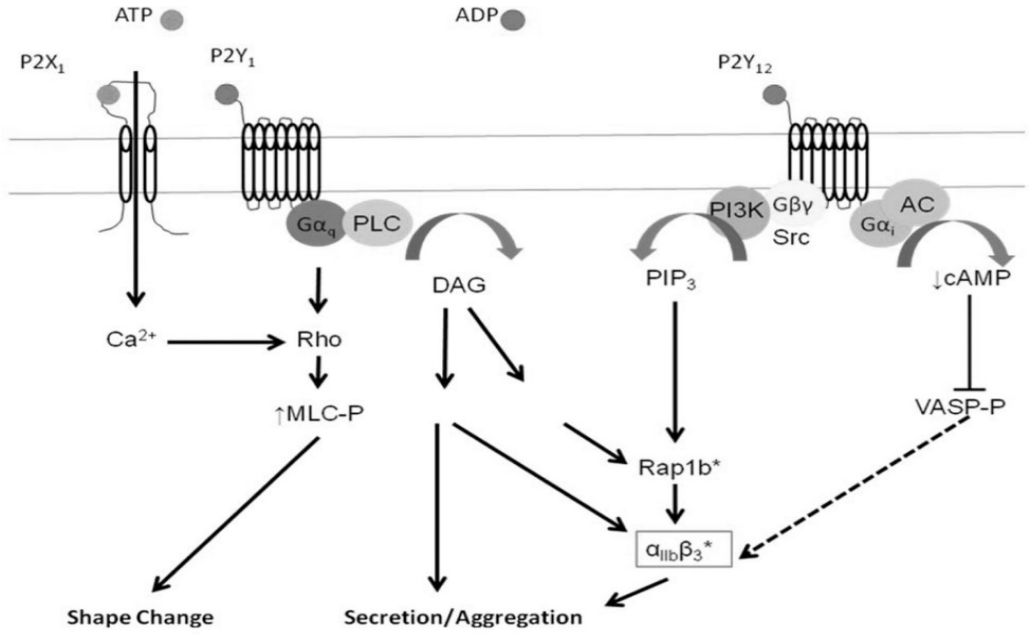

Figure 1.7: Signalisation des récepteurs purinérgiques. Cunningham MR et al. *Biochem Soc Trans*. 2013 Feb 1; 41(1):225-30

1.2.3.4 $P2X_1$

Le récepteur $P2X_1$ est le seul canal ionique activé par l'ATP sur la surface plaquettaire (112). Suite à son activation par l'ATP, le $P2X_1$ induit un influx rapide de Ca^{2+} qui engendre l'activation de différentes protéines y compris la protéine Rho et MLCK (*Myosin Light Chain Kinase*) qui phosphoryle la MLC (*Myosin Light Chain)*, un processus qui contribue au changement de forme plaquettaire et la relâche de granules (113). Cependant, cela ne peut déclencher une agrégation. Par contre, et surtout en présence de force de cisaillement élevée,

$P2X_1$ participe de façon importante à l'amplification de l'agrégation en coopération avec d'autres agonistes plaquettaires comme le collagène (114-116).

1.2.3.5 PARs

L'activation plaquettaire par la thrombine est initiée *via* le GPIb-IX-V **(**voir **section 1.2.1)**, mais elle est surtout assurée par les PARs. Il y a quatre membres de la famille des PAR (PAR1-4). Le PAR1 et le PAR4 (expression négligeable du PAR3) sont exprimés à la surface de plaquettes humaines; or, les souris expriment les membres PAR3 et PAR4. Le PAR2 est présent à la surface d'autres types cellulaires comme les cellules endothéliales, mais pas sur les plaquettes (117-119). Chaque PAR a une expression membranaire d'environ 1500 à 2000 copies par plaquette (120). Le PAR1 est le premier récepteur de la famille des PARs à être découvert dans les plaquettes humaines et il a une forte affinité à la thrombine comparée au PAR4 qui demande des plus fortes concentrations de thrombine pour être activé (121, 122). Suite à leur liaison à la thrombine, les PARs plaquettaires possèdent un mécanisme d'action distinct des autres GPCR, qui consiste en un clivage irréversible de l'extrémité N-terminale extracellulaire des PARs par la thrombine ainsi qu'en exposant une nouvelle extrémité N-terminale qui se replie et agit en tant qu'autoagoniste (Figure 1.8) (121). Une fois activés, ils subissent une endocytose afin d'être dégradés par les lysosomes cytoplasmiques (123, 124). Les PAR1 et PAR4 sont couplés à des protéine G_q et $G_{12/13}$ qui induisent l'activation d'une panoplie de protéines(125). D'une part, l'activation des protéines G_q mène à la phosphorylation du PLCβ qui conduit à l'activation des isoformes des PKC et la libération du calcium intracellulaire *via* la génération du DAG et l'IP3 respectivement. D'une autre part, l'activation du $G_{12/13}$ induit l'activation de Rho kinase et du MLC (97, 126). De plus, PAR1 est couplé à la protéine G_i qui bloque l'AC et la production du AMPc (122, 127). De ces cascades de signalisation découlent les différents effets de la thrombine sur les PARs plaquettaire où on assiste à une amplification de l'activation et de l'agrégation plaquettaire suite à leur changement de forme et relargage des contenus des granules ainsi que la production de TXA_2 par la COX-1, l'activation des intégrines $\alpha_{IIb}\beta_3$ *via* le mécanisme de «*inside-out*» et l'augmentation de l'activité procoagulante des plaquettes en exposant les phospholipides procoagulants (122, 128, 129). Des patients présentant une déficience congénitale des PARs n'ont pas été identifiés. Cependant, des modèles de souris déficientes de PAR3 ou PAR4 exhibent un temps de saignement prolongé et une protection contre la thrombose artérielle (130-133).

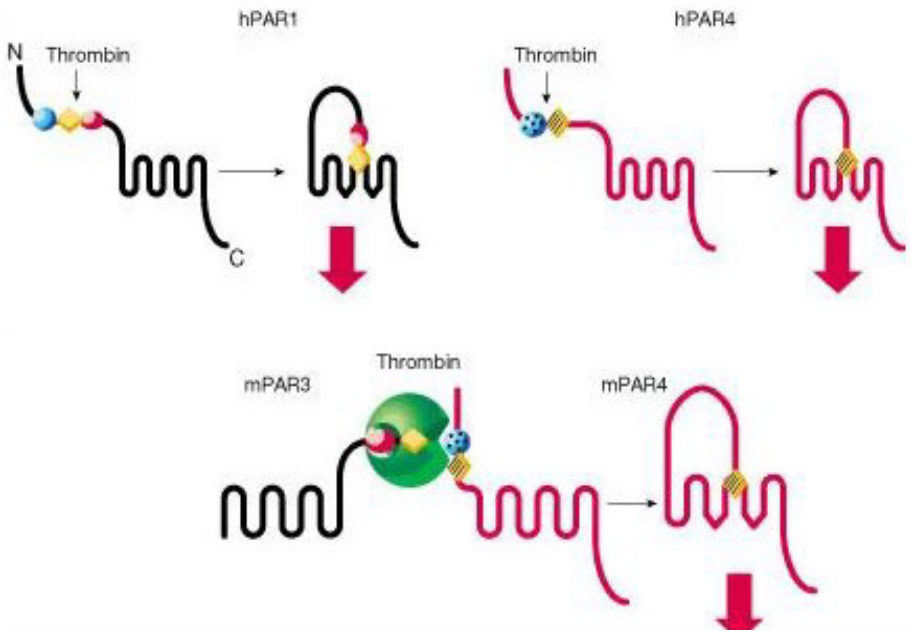

Figure 1.8: l'action de la thrombine chez les plaquettes humaines et souris. Coughlin SR *Nature.* 2000 Sep 14;407(6801):258-64.

1.3 Fonction plaquettaire

Les plaquettes jouent un rôle essentiel dans la régulation de la thrombose et l'hémostase. Ainsi, suite à une lésion vasculaire, les plaquettes s'activent en adhérant à la matrice extracellulaire exposée du sous-endothélium et induisent la formation d'un clou plaquettaire et la consolidation du thrombus afin de maintenir l'intégrité du vaisseau sanguin. Notre compréhension de l'ampleur et de la complexité des étapes qui régissent la fonction plaquettaire s'est rapidement développée au cours de la dernière décennie (134-137). Ces étapes sont représentées en quatre événements successifs, soit l'adhésion, l'activation, la sécrétion et l'agrégation plaquettaire. Ainsi, on discutera dans cette section de l'implication de la signalisation plaquettaire exploitée dans la section 1.2 au niveau de ces évènements.

1.3.1 Adhésion plaquettaire

Dans des circonstances physiologiques normales, les plaquettes circulent dans le sang dans un état inactif; cela est dû en grande partie à l'action de l'endothélium *via* plusieurs mécanismes, dont l'expression par les cellules endothéliales de ENTPD1 (*Ectonucleoside triphosphate diphosphohydrolase 1*) dégradant l'ATP et l'ADP ainsi que la thrombomoduline qui inactive la thrombine (138). De plus, ils sécrètent le monoxyde d'azote (*Nitric oxide* ou NO) et la prostaglandine I_2 (PGI_2, ou prostacycline) qui génèrent la production de la guanosine monophosphate cyclique (GMPc) et AMPc *via* la stimulation de la guanylate cyclase (GC) et AC dans les plaquettes, inhibant ainsi leur activation (139). Cependant, suite à une lésion du vaisseau sanguin, l'action de l'endothélium est altérée et des composantes sous-endothéliales sont exposées dont le VWF, le collagène, la fibronectine et la laminine, qui interagissent avec des récepteurs d'adhésion de la membrane plaquettaire. Dès lors, les plaquettes roulent sur de l'endothélium activé grâce à l'expression membranaire de la P-séléctine provenant des corps de Wiebel-Palade des cellules endothéliales activées et sa liaison avec le PSGL-1 plaquettaire ainsi que le VWF exposé sur la matrice extracellulaire de l'endothélium et le GPIb-IX-V (140).

L'interaction des plaquettes avec le sous-endothélium dépend de l'hémodynamie au sein du vaisseau. D'une part, lorsque les forces de cisaillement sont élevées, telle que retrouvées dans les artères sténosées où le flux sanguin est rapide, l'interaction initiale des plaquettes avec le sous-endothélium est médiée par le VWF. En effet, le VWF multimérique circulant est d'abord clivé par ADAMTS-13 permettant la réducation de la taille de ces multimères, suite à cela, l'exposition du collagène au niveau de l'endothélium s'associe au VWF circulant. Cette association induit un changement conformationnel du VWF, le rendant capable de lier le GPIb-IX-V plaquettaire (141). Malgré que cette interaction n'entraine pas d'adhésion ferme, elle est néanmoins la plus cruciale pour le recrutement des plaquettes au sein de la lésion vasculaire et leur ralentissement afin qu'elle adhère solidement à la paroi *via* le GPVI et $\alpha_2\beta_1$ (142). D'autre part, lorsque les forces de cisaillement sont faibles et donc le flux sanguin est lent comme dans un réseau veineux, le rôle du GPIb-IX-V est moins important dans l'adhésion plaquettaire; elle ainsi surtout dépendante des récepteurs GPVI et $\alpha_2\beta_1$ (143). Par conséquent, l'adhésion stable des plaquettes à la matrice sous-endothéliale se fait *via* les récepteurs GPVI et $\alpha_2\beta_1$ avec le collagène exposé, mais aussi *via* l'intégrine $\alpha_5\beta_1$ avec la fibronectine, l'intégrine $\alpha_5\beta_1$ avec la

laminine, le CD36 avec la thrombospondin-1 (TSP-1) et l'intégrine $\alpha_{IIb}\beta_3$ qui se lie à plusieurs récepteurs endothéliaux dont l'$\alpha_v\beta_3$ endothélial (*via* le VWF, le fibrinogène, la fibronectine), ICAM-1 (*via* fibrinogène) et GPIb-IX-V (*via* le VWF) (Figure 1.9) (140).

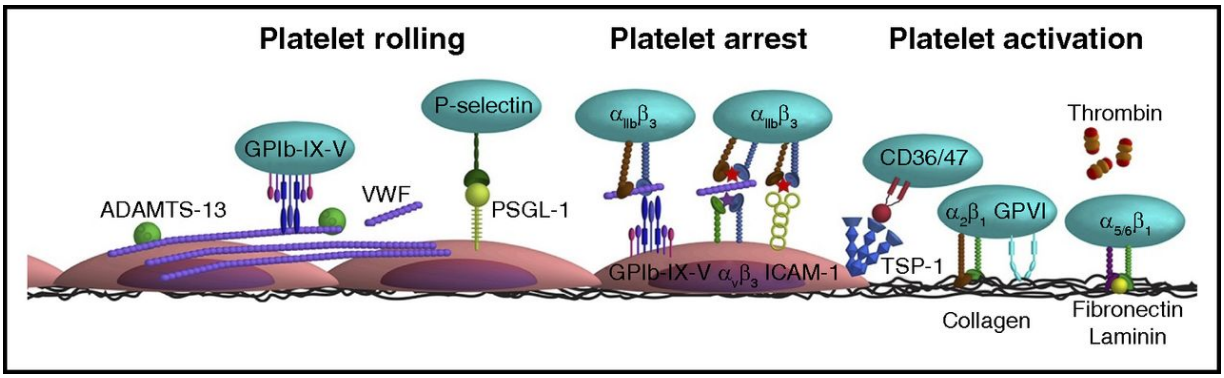

Figure 1.9: Représentation des interactions entre les plaquettes et la surface endothéliale intacte et endommagée. Daniëlle M. Coenen et al. *Blood* 2017;130:2819-2828

1.3.2 Activation plaquettaire

Suite à l'adhésion des plaquettes *via* les récepteurs d'adhésion plaquettaire tels que le GPIb-IX-V, le GPVI, $\alpha_2\beta_1$, ces mêmes récepteurs induisent l'activation plaquettaire qui consiste en un changement de forme des plaquettes, la sécrétion plaquettaire et l'activation des intégrines. Comme mentionné dans la section 1.2, la signalisation de ces récepteurs consiste par l'activation de la tyrosine kinase de la famille Src, qui à son tour active le PLCγ2 menant à la génération de l'IP_3 et DAG qui entrainent l'activation des isoformes de PKC et la mobilisation du Ca^{2+} intracellulaire. Cette signalisation mène au changement de forme des plaquettes et leur étalement par l'émission des filopodes et lamellipodes, la relâche des granules *via* les SNAREs (*soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors*) (voir **section 1.3.3**) et l'activation «*inside-out*» des intégrines dont l'$\alpha_{IIb}\beta_3$ favorisant sa liaison au fibrinogène, une étape primordiale pour l'agrégation plaquettaire. Enfin, la mobilisation du Ca^{2+} cytosolique enclenche aussi l'exposition de la phosphatidylsérine (PtdSer) à la membrane plaquettaire promouvant l'augmentation de la charge négative au niveau de la membrane (144). Cette exposition de la PtdSer est médiée par l'anoctamin-6 (ANO6) et la protéase intracellulaire, la calpain 2 (145, 146). La PtdSer constitue une surface pro-coagulante pour les facteurs de

coagulation accélérant ainsi la cascade de la coagulation. Ce processus est soutenu par la libération de microparticules plaquettaires (MP) exposant à leur surface la PtdSer et la libération de facteurs tissulaires qui assiste la coagulation sanguine favorisant l'élévation des niveaux de thrombine circulant au sein de la lésion (147). L'importance de la PtdSer est soulignée dans le syndrome de Scott où les patients souffrent d'un syndrome hémorragique dû à un défaut de translocation de la PtdSer, qui résulte en un arrêt prématuré de la protéine (144, 146).

L'initiation de l'activation plaquettaire suite à leur adhésion à la matrice sous-endothéliale provoque une sécrétion des médiateurs secondaires dont notamment le TxA_2 et l'ADP qui, en agissant sur leur récepteur TP et les récepteurs purinérgiques respectivement, amplifient l'activation plaquettaire. Parallèlement, l'expression du TF par les cellules recrutées au site de la lésion vasculaire telle que les monocytes, leucocytes, cellules endothéliales et plaquettes activées engendre une génération importante de thrombine qui participe au phénomène d'amplification de l'activation plaquettaire en agissant sur les récepteurs de thrombine exprimés sur les plaquettes - plus précisément, les PARs (148). Ces récepteurs d'amplification sont couplés à la protéine G (voir **section 1.2.3**). Il existe trois voies essentielles signalétiques impliquées dans l'activation de ces récepteurs, soit celles provenant de l'activation des protéines G_q, $G_{12/13}$ et G_i (149). D'abord, la stimulation de la protéine G_q induit l'activation de la PLCβ; cette voie engendre l'activation des isoformes de PKC et la libération du Ca^{2+} intracellulaire *via* la génération du DAG et de l'IP3. Cette signalisation est primordiale pour l'activation des intégrines menant à l'agrégation des plaquettes. Ensuite, la stimulation de la protéine $G_{12/13}$ induit l'activation du RhoGEF qui mène à l'activation de RhoA et la Rho kinase (ROCK) qui conduit à la phosphorylation du MLC, une étape primordiale pour le changement de forme et le réarrangement cytosquelettique des plaquettes. Enfin, la stimulation de la protéine G_i entraine l'inhibition de l'AC et, par conséquent, bloque la génération de l'AMPc permettant l'inhibition de la voie impliquée dans l'inactivation des plaquettes et ainsi mène à l'activation des intégrines permettant l'activation et l'agrégation plaquettaire (136, 149). La Figure 1.10 résume les voies de signalisation essentielles à l'activation plaquettaire.

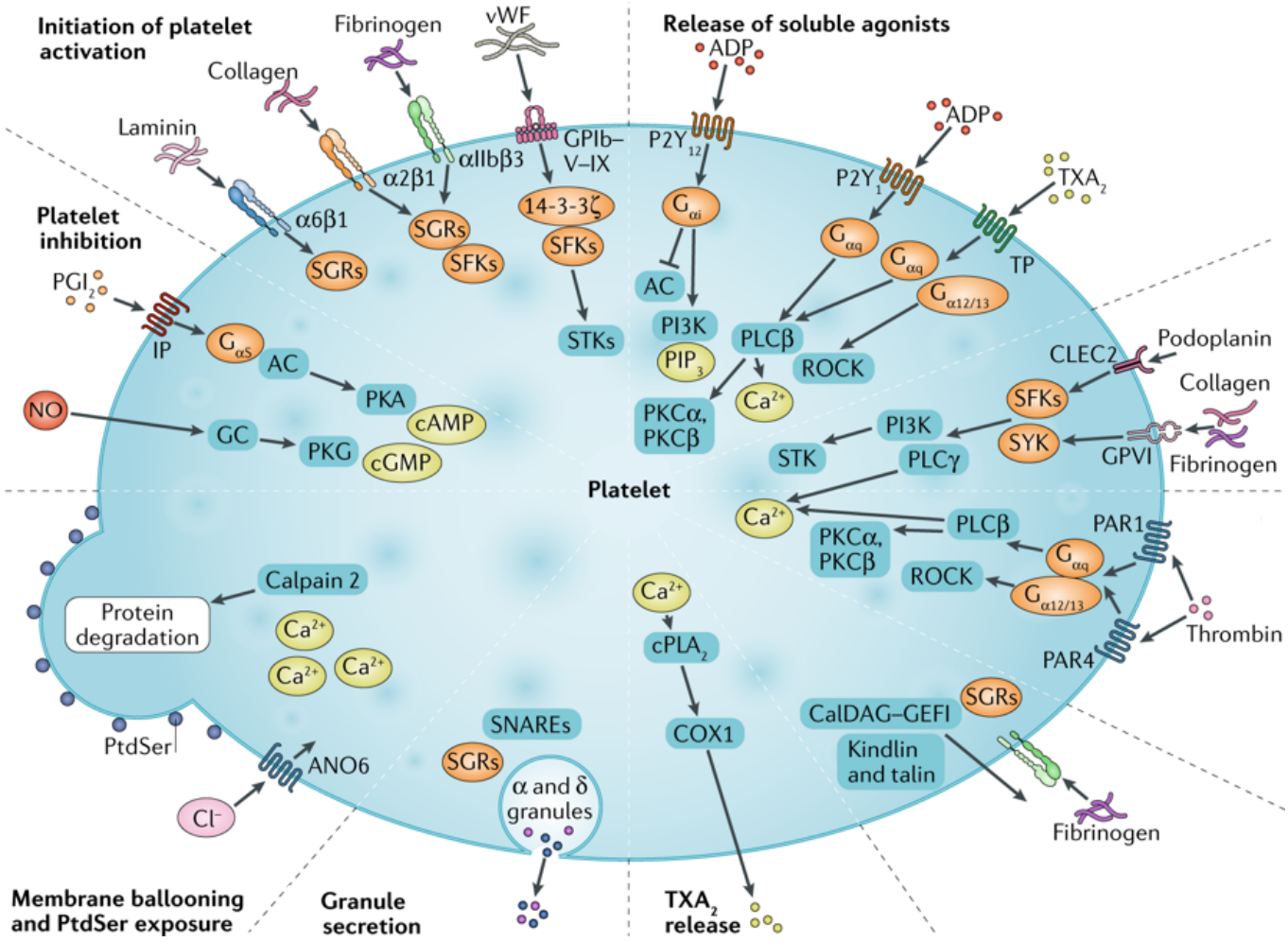

Figure 1.10: Les voies de signalisation majeure des événements impliqués dans l'activation plaquettaire. Van der Meijden. PEJ et al. *Nat Rev Cardiol.* 2019 Mar;16(3):166-179.

1.3.3 Sécrétion plaquettaire

L'activation plaquettaire provoque la sécrétion d'une panoplie de protéines contenues dans des granules ou des protéines nouvellement synthétisées comme le TxA_2. La sécrétion plaquettaire est primordiale dans le rôle pathophysiologique des plaquettes; en effet, ce processus permet, parmi d'autres, le recrutement et l'activation des plaquettes circulantes et localisées au site de lésion ainsi que la migration d'autres types de cellules comme les leucocytes au sein de la lésion et la formation du thrombus et sa stabilisation. Les plaquettes contiennent trois types de granules, soit les α-granules, les granules denses et les lysosomes. Les granules

des plaquettes ont d'abord été identifiées à la fin du XIX^{ème} siècle; cependant, il fallait attendre jusqu'à 1966 afin de pouvoir discerner les granules denses des α-granules (150) et une année de plus pour distinguer les lysosomes grâce à la mise au point de la microscopie électronique (151). La formation des granules débute au sein des mégacaryocytes et mature dans les plaquettes circulants (152-154). Des études protéomiques ont identifié environ 800 protéines contenues dans les granules plaquettaires (155-157). Le tableau 1.1 résume les protéines principales contenues dans ces granules.

1.3.3.1 Les α-granules

Les α-granules sont les granules les plus larges et les plus abondants dans les plaquettes avec environ 50 à 80 α-granules par plaquette et ont souvent une forme sphérique de 200 à 500 nm de diamètre et occupent 10% du volume des plaquettes. Leur développement débute dans les mégacaryocytes et se suit dans les plaquettes *via* les corps multi-vésiculaires qui sont constitués par les vésicules fragmentées du réseau trans-Golgi au niveau des mégacaryocytes (158, 159). Ils contiennent des protéines qui s'associent à la membrane et des protéines solubles, qui sont impliquées dans plusieurs processus dont l'adhésion et le recrutement cellulaire comme le VWF et la thrombospondine, la coagulation comme le facteur V et facteur IX ainsi que des protéines pro-inflammatoires telles que les chimiokines et les facteurs de croissance (exemples : CD40L, P-séléctine, PF4, IL-1β, RANTES, MCP-1, MIP-1α, VEGF, et PDGF) (155, 158, 160). Suite à l'activation plaquettaire, des protéines peuvent s'exprimer à la surface des plaquettes ou être relâchées dans le milieu extracellulaire. Plusieurs des protéines présentes dans les α-granules sont exprimées sur la surface plaquettaire quand les plaquettes sont en repos, comme les intégrines tels que les $\alpha_{IIb}\beta_3$, les immunoglobulines telles que le GPVI et FcR (161), et autres récepteurs d'adhésion comme le complexe GPIb-IX-V(160). D'autres récepteurs en revanche sont exprimés suite à l'activation des plaquettes à la surface des plaquettes comme le CD40L et la P-séléctine, les rendant des biomarqueurs de l'activation plaquettaire (148, 155). Enfin, la contribution des α-granules à l'hémostase est observée chez les patients souffrant du syndrome des plaquettes grises. Chez ces patients, on observe un syndrome hémorragique modéré dû à une thrombocytopénie; cette hémorragie est souvent accompagnée d'une myélofibrose et d'une splénomégalie (162). Le gène qui est souvent impliqué dans ce syndrome est le NBEAL2; des mutations au niveau de ce gène altèrent la biogenèse des α-granules (163).

1.3.3.2 Les granules denses

Les granules denses sont moins abondants et volumineux que les α-granules, avec environ 3 à 8 granules denses par plaquette avec un diamètre compris entre 150 à 300 nm. Similairement aux α-granules, le développement des corps multi-vésiculaires au niveau des mégacaryocytes est une étape menant aux maturations des granules denses (159). Ils contiennent une concentration élevée en ADP, ATP qui sont essentielles à l'amplification de l'activation plaquettaire, ainsi que des cations comme le Ca^{2+}, Mg^{2+} et K^{+} et autres constituants tels que la sérotonine, l'histamine et des phosphatases contribuant à la formation du clou hémostatique (164-166). Le rôle des granules denses est démontré chez les patients souffrant du syndrome d'Hermansky-Pudlak qui se caractérise par des troubles hémorragiques dus à une faible agrégation plaquettaire secondaire à l'absence des granules denses dans les plaquettes. Ce syndrome est causé par des mutations des gènes HPS; ces mutations sont aussi associées à un albinisme oculocutané et la présence d'une fibrose pulmonaire chez ces patients (167, 168).

1.3.3.3 Les lysosomes

Les lysosomes plaquettaires sont des vésicules sphériques qui sont les moins abondantes, d'un diamètre de 175 à 250 nm et présentant un aspect morphologique similaire aux α-granules; ils sont au nombre de 1 à 3 lysosomes par plaquette (159, 169). Ils contiennent surtout des enzymes protéolytiques de dégradations qui contribuent aux remodelages du thrombus tels que des protéinases comme les cathepsines, l'élastase et la collagénase ainsi que d'autres enzymes de dégradation comme les glucosidases, les galactosidase et phosphatase d'acide (159).

Tableau 1.1. Principaux contenus des granules plaquettaires.

	α granules	**Granules denses**	**Lysosomes**
Diamètre	200-500 nm	150-300 nm	175-250 nm
Nombre/plaquette	50-80	3-8	< 3
Fonctions générales	Hémostase/thrombose Inflammation Angiogenèse Mitogenèse Réponse immunitaire	Hémostase/thrombose Inflammation	Digestion des endosomes
Contenu des granules	$\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$ GPIb-IX-V, GPVI, FcR TLT-1 P-séléctine CD40L Fibrinogène Fibronectine Vitronectine Ostéonectine VWF, VWF antigen II Thrombospondine GRO-α PF4 ENA-78 PBP β-TG NAP-2 IL-1, IL-1α, IL-1β, IL-7, IL-8 SDF-1α MCP-1 MIP-1α RANTES TGF-β IGF, HGF, EGF, FGF, VEGF, PDGF ADAM10, ADAMST13 MMP-1, MMP-2, MMP-9 Facteur V-VIII-IX-XI-XIII Plasminogène Protéine S PAI-1 α2-antiplasmine α2-antitrypsine α2-macroglobuline IgG, IgA, IgM Thrombocidine 1-2 c3/c4 précurseur C1-inhibiteur	CD107a (LAMP-1) CD107b (LAMP-2) CD63 (LAMP-3) Sérotonine Histamine ATP ADP GTP GDP UTP Phosphate Pyrophosphate Ca^{2+} Mg^{2+} K^+	Cathepsine D Cathepsine E Elastase Collagénase Carboxypeptidase A Carboxypeptidase B Carboxypeptidase β-N-acétyl-D hexosaminidase β-D-glucuronidase β-D-galactosidase α-D-mannosidase α-L-arabinofuranosidase α-D-galactosidase α-L-fucosidase β-D-fucosidase β-D-glucosidase α-D-glucosidase Phosphatase acide Arylsulphatase

1.3.4 Agrégation plaquettaire

L'agrégation plaquettaire est l'étape finale dans l'hémostase primaire menant à la formation d'un clou hémostatique suite à l'activation des plaquettes. Cette étape est engendrée par le changement de conformation de l'intégrine $\alpha_{IIb}\beta_3$ et sa liaison au fibrinogène soluble (pour la structure voir section 1.2.2.5). Ainsi, l'activation plaquettaire, par ses agonistes telle que la collagène, la thrombine ou l'ADP, induit une cascade de voies signalétiques entrainant la disjonction des queues cytoplasmiques des sous-unités α et β, permettant alors le changement conformationnel de l'intégrine $\alpha_{IIb}\beta_3$ afin d'augmenter son affinité de liaison au fibrinogène (170). Ce mécanisme implique plusieurs partenaires de liaison au niveau de la queue cytoplasmique dont notamment le Rap1b, la taline et la kindlin-3 permettant l'activation de l'intégrine, un processus appelé la signalisation *«inside-out»* (Figure 1.5) (50). Lorsque le fibrinogène s'associe à l'intégrine $\alpha_{IIb}\beta_3$, elle induit une cascade signalétique appelée *«outside-in».* Ainsi, le changement conformationnel de l'intégrine permet le recrutement des Src kinase qui s'active par autophosphorylation. La calpaine clive la queue cytoplasmique de la sous-unité β_3, menant à la dissociation de la Src kinase active de la queue. Ainsi, elle phosphoryle une panoplie d'enzymes et des protéines de signalisation dont FAK, Syk kinase, RhoGAP, Rac-GEF et le PI3K. Ces signalisations permettent l'ancrage et l'étalement plaquettaire aux protéines de la matrice sous-endothéliale exposée au niveau de la lésion. En effet, ces signalisations impliquent aussi la protéine $G\alpha_{13}$, la taline, la kindlin-3, la tensine et la vinculine afin d'assurer la liaison nécessaire entre la queue cytoplasmique de la sous-unité β_3 à l'actine du cytosquelette. Cette liaison de la sous-unité β_3 à l'actine du cytosquelette est renforcée par le complexe ILK/PINCH/Parvin (Figure 1.11) (78). L'agrégation plaquettaire et l'activation du système de coagulation humorale sont au centre du stade final de la formation du thrombus.

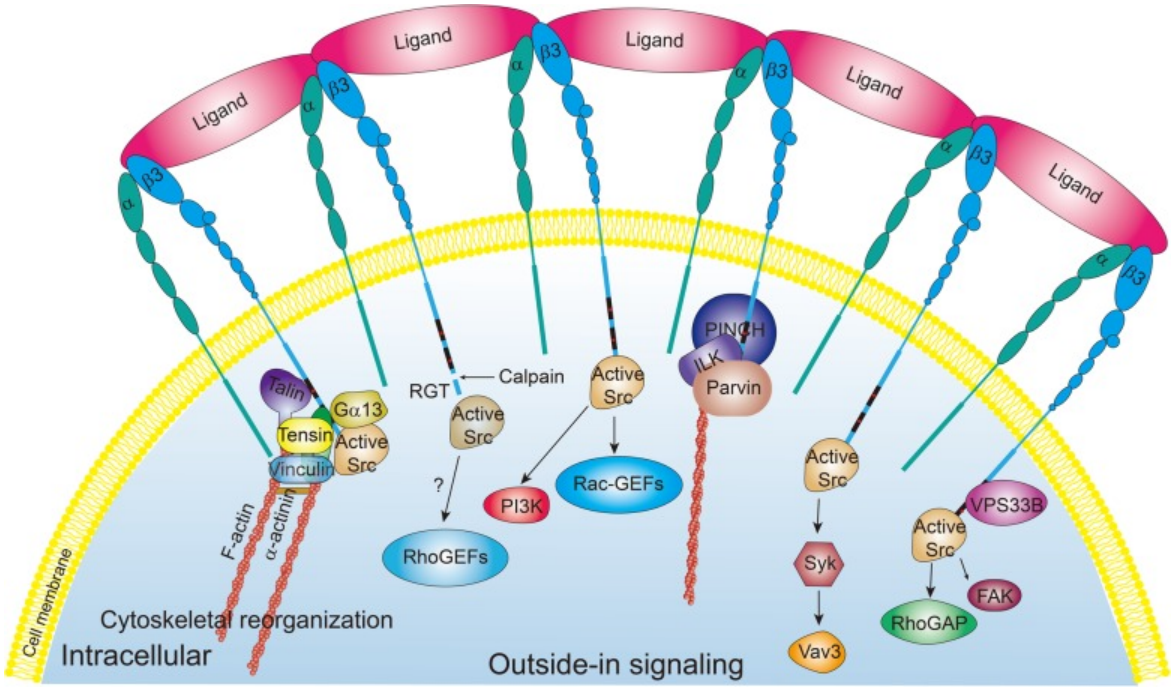

Figure 1.11: Signalisation *«outside-in»* de l'intégrine $\alpha_{IIb}\beta_3$. Huang. J et al. *J Hematol Oncol.* 2019 Mar 7; 12(1):26.

1.4 Plaquettes et maladies cardiovasculaires

Les maladies cardiovasculaires représentent la plus grande cause de mortalité globalement. Selon le rapport de l'Organisation mondiale de la santé (OMS), le taux de mortalité causé par les maladies cardiovasculaires dans le monde est estimé à 17.3 millions de personnes en 2008, représentant 30% des décès globalement. De plus, il est estimé que ce nombre risque d'augmenter jusqu'à un nombre atteignant 23.6 millions de personnes (171). Les maladies cardiovasculaires sont souvent causées par des évènements thrombotiques tels que les maladies coronariennes. Les plaquettes jouent différents rôles dans les maladies cardiovasculaires. En effet, comme on l'a vu dans la section précédente, elles sont les joueuses principales dans l'hémostase primaire et la formation de la thrombose; cependant, ce phénomène de thrombose peut devenir pathologique quand il a lieu, par exemple, suite à la rupture d'une plaque athérosclérotique. Ainsi, la formation d'un thrombus peut entrainer, dans un cas pathologique, une occlusion partielle ou complète du vaisseau sanguin menant ainsi à une diminution ou blocage du flux sanguin et devient ainsi une cause d'occurrence d'ischémie ou d'infarctus d'un organe irrigué. De plus, les plaquettes contribuent à l'initiation et l'accélération de l'inflammation par leur sécrétion d'une panoplie de molécules pro-inflammatoires telles que les

cytokines et les chimiokines. Leur capacité de participer à l'inflammation fait d'eux des complices dans des pathologies à caractère inflammatoire tel l'athérosclérose.

1.4.1 Thrombose

L'occurrence d'une thrombose se manifeste suite à la formation incontrôlée d'un clou qui occlue partiellement ou complètement un vaisseau sanguin dû à un déséquilibre au niveau de l'hémostase. La présence des plaquettes et les facteurs de coagulations jouent un rôle primordial dans la formation d'un thrombus. La pathophysiologie de la thrombose diffère dépendamment que ce soit au niveau d'une artère ou d'une veine. D'abord, les syndromes coronariens aigus (SCA), l'infarctus du myocarde (IM) et les accidents vasculaires cérébraux (AVC) sont surtout causés par les thrombus artériels; or, la thrombose veineuse profonde (TVP) et l'embolie pulmonaire (EP) sont causées par le thrombus veineux. Ensuite, la structure et la causalité du thrombus diffèrent entre les deux types de vaisseaux. En effet, le thrombus artériel est riche en plaquettes et se forme surtout au site où les forces de cisaillement sont élevées et il y a occurrence d'une rupture d'une plaque athérosclérotique due à l'accumulation de dépôts lipidiques et de cellules spumeuses (172). Les étapes de l'hémostase et aussi la formation du thrombus plaquettaire pathologique sont décrites dans la section 1.3; brièvement, la rupture de la plaque entraine l'exposition du collagène de la matrice sous-endothéliale. Ainsi, les plaquettes adhérent grâce à leurs récepteurs, tels que les GPVI et le GPIb-IX-V qui se lient au collagène et le VWF, respectivement et forment une monocouche plaquettaire. Suite à cela, les plaquettes activées forment une structure trois dimensionnelle en agrégeant, *via* l'activation de l'intégrine $\alpha_{IIb}\beta_3$. Ce phénomène d'agrégation plaquettaire est amplifié par le clivage des PARs par la thrombine qui est activée par la cascade de coagulation sanguine initiée par le TF présent en concentration élevée au site athérosclérotique et par la sécrétion des contenus des plaquettes tels que l'ADP et le TxA_2. Ainsi, les thrombus artériels sont surtout traités par des thérapies qui ciblent l'activation et l'agrégation plaquettaire (voir chapitre 4). L'activation de la thrombine mène à la génération de la fibrine, une constituante principale en commun entre le thrombus artériel et veineux. Cependant, la formation du thrombus veineux diffère du thrombus artériel, qui peut avoir lieu même si l'endothélium demeure intact. En effet, il est surtout entrainé par un changement dans la composition du sang, comme dans le cas d'une thrombophilie, affectant ainsi le flux sanguin en le ralentissant ou le stagnant, favorisant l'accumulation de protéases procoagulantes telle que la thrombine. D'ailleurs, à l'opposé de l'artère, les forces de

cisaillement sont basses au niveau des vaisseaux veineux. Ainsi, le thrombus veineux est riche en fibrine entremêlée avec des globules rouges et des plaquettes, d'où le nom, thrombus rouge (en revanche le thrombus riche en plaquettes qui se forme dans l'artère se nomme thrombus blanc). Ainsi, la cascade de coagulation est au centre de la formation du thrombus veineux. L'activation de l'endothélium veineux causée par l'inflammation ou des conditions d'hypoxie suite au ralentissement ou stagnation du flux sanguin incite l'expression du TF, de la P-séléctine et du VWF. Ce processus induit le recrutement de plaquettes et de leucocytes à l'endothélium actif, déclenchant ainsi une amplification de la cascade de coagulation par l'augmentation de l'expression du TF. De plus, les globules rouges contribuent à la maintenance du thrombus veineux en empêchant le mécanisme de fibrinolyse par leur capacité à changer d'une forme biconcave à une forme polyédrique. Ainsi, les traitements du thrombus veineux ciblent plutôt les protéines qui régissent la cascade de coagulation. Finalement, les thrombus veineux adhérent moins à l'endothélium comparé aux thrombi artériels, et peuvent ainsi être plus facilement délogés dans la circulation et entrainer des occlusions vasculaires comme dans le cas de l'embolie pulmonaire. La Figure 1.12 résume les différences discutées entre la thrombose artérielle et la thrombose veineuse.

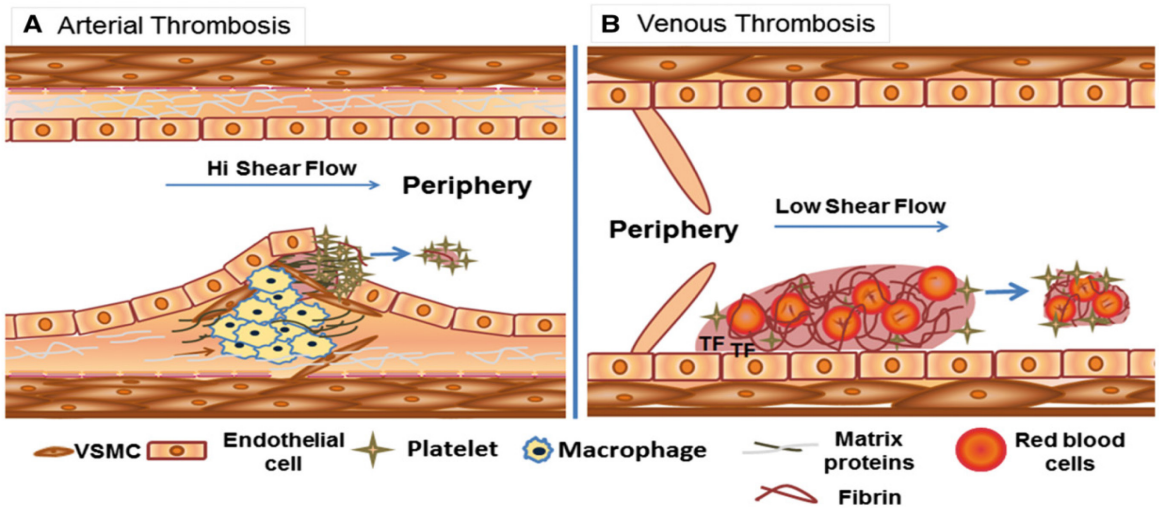

Figure 1.12: Les différences majeures entre la thrombose artérielle et la thrombose veineuse. Koupenova M et al. *Eur Heart J*. 2017 Mar 14;38(11):785-791.

1.4.2 Athérosclérose

La pathogenèse de l'athérosclérose englobe un grand nombre de constituants inflammatoires impliquant les plaquettes et leurs médiateurs d'inflammations (173-175).

Auparavant, la contribution pathophysiologique des plaquettes à l'athérosclérose s'arrêtait à la fonction hémostatique des plaquettes dans l'étape finale de l'athérosclérose ainsi que leur fonction thrombotique suite à la rupture de la plaque athérosclérotique. Aujourd'hui, on leur attribue aussi un rôle dans l'athérogenèse grâce à leur capacité à orchestrer la réaction inflammatoire et le recrutement des cellules qui incitent la progression de l'athérosclérose (Figure 1.13) (176-178). En effet, malgré que l'endothélium ne soit pas encore lésé pour induire l'adhésion plaquettaire, l'activation des cellules endothéliales déclenchent l'expression des molécules comme la P-séléctine et le VWF, permettant l'adhésion des plaquettes *via* des récepteurs d'adhésion comme GPIb-IX-V et PSGL-1 et leur activation (179, 180). L'activation plaquettaire entraine le relâchement d'une panoplie de médiateurs pro-inflammatoires, comme des cytokines et chimiokines (CD40L, IL-1β, RANTS, PF4, SDF-1, MCP-1, et autres). Par conséquent, il y a une amplification de la réaction inflammatoire au site de formation de la plaque athérosclérotique et au lieu de recrutement des leucocytes, monocytes et cellules endothéliales progénitrices, ce qui favorise l'altération de l'endothélium enflammé, une étape cruciale pour la formation de la lésion athérosclérotique (176, 181-183). De plus, les plaquettes participent à la différentiation des monocytes en des cellules spumeuses (ou macrophages spumeux) et cela, par leur capacité à transporter le LDL oxydé (oxLDL) en les capturant et phagocytant *via* les récepteurs éboueurs ou *« scavenger »* -dont les CD36, CD68, LOX-1 et PS-OX. Ainsi, les monocytes recrutés par les médiateurs pro-inflammatoires plaquettaires phagocytent les oxLDL des plaquettes et se différencient en des cellules spumeuses (184-186). L'accumulation des cellules spumeuses est une étape primordiale à la formation de la plaque athérosclérotique. En tout, l'implication des plaquettes dans les mécanismes inflammatoires dans l'athérosclérose transcende son rôle classique dans la thrombose et l'hémostase suite à la rupture de la plaque athérosclérotique.

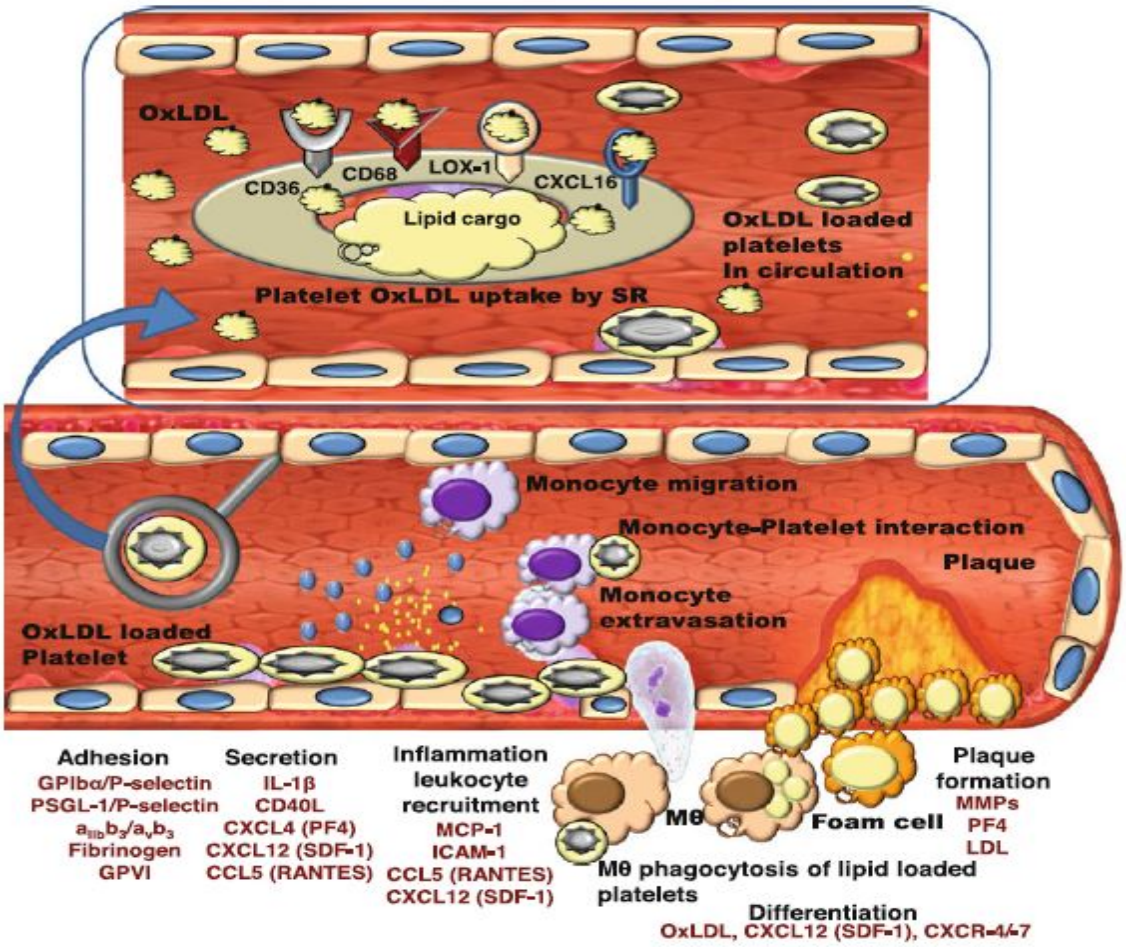

Figure 1.13: Le rôle des plaquettes dans l'athérogenèse. M. Chatterjee et M. Gawaz, chapitre: Platelets in Atherosclerosis, page 944. Platelets in thrombotic and non-thrombotic disorders. *Springer Nature* Mar 2017.

Chapitre 2 : Le CD40L

2.1 Origine

Le CD40L (aussi appelé CD154, gp39, TRAP et TBAM) est une protéine transmembranaire de type II appartenant à la superfamille des TNF (*Tumour necrosis factor*). Le gène codant du CD40L se trouve dans la région Xq26-q27 du chromosome X, la taille du fragment est de 12 à 13 kb. Ce fragment est composé de cinq exons; ainsi, la région intracellulaire et transmembranaire du CD40L est codée par l'exon I. Or, la région extracellulaire est codée par les exons II-V.

Le CD40L a été d'abord identifié chez les lymphocytes T $CD4^+$; on lui a attribué un rôle unique de modulateur immunitaire, et cela en interagissant avec un de ses récepteurs, le CD40, exprimé sur les lymphocytes B, où il entraine la prolifération et la différenciation des lymphocytes B. Effectivement, l'importance du CD40L dans l'immunité est soulignée chez les patients atteints du syndrome d'hyper-IgM lié à l'X (HIGM) qui présentent une absence de la fonction du CD40L dû à une mutation au niveau de son gène. Par conséquent, on observe chez ces individus une élévation des niveaux d'IgG et une absence de IgG, IgA et IgE circulants causée par l'absence du rôle crucial du CD40L dans la commutation isotypique du IgM en d'autres isotypes d'immunoglobulines. Le défaut au niveau de la commutation isotypique engendre chez ces patients une immunodéficience qui se caractérise par une importante susceptibilité d'infections bactériennes récurrentes, y compris des infections respiratoires et des pneumonies, ainsi que des risques de développer des maladies auto-immunitaires et lymphoprolifératives et des neutropénies. Cependant, des études durant les années qui suivent la découverte du CD40L démontrent que son expression n'est pas qu'exclusive sur les lymphocytes T, mais d'autres cellules du système vasculaire l'expriment aussi. Dès lors, la fonction du CD40L transcende la simple fonction d'un régulateur immunitaire et participe activement dans diverses physiopathologies impliquant un environnement inflammatoire comme l'athérosclérose. Aujourd'hui, le CD40L est perçu comme un candidat thérapeutique pour une panoplie de pathologies dont les maladies cardiovasculaires suite à l'émergence des études qui relient l'apparition de ces pathologies au niveau de CD40L soluble (sCD40L) circulant.

2.2 La structure du CD40L et ses récepteurs

Le CD40L est une protéine de 39 kDa; cependant, ce poids moléculaire est variable dû à des modifications post-traductionnelles (187). Il est constitué de 261 acides aminés et comprend un domaine C-terminal situé dans la région extracellulaire de 215 acides aminés, un domaine N-terminal dans la région intracellulaire de 22 acides aminés et un domaine transmembranaire de 24 acides aminés (188). Malgré que le CD40L soit une protéine de type II transmembranaire, elle forme un complexe multimérique composé de trois monomères (trimère) associés sur la membrane cellulaire (Figure 2.1) (189).

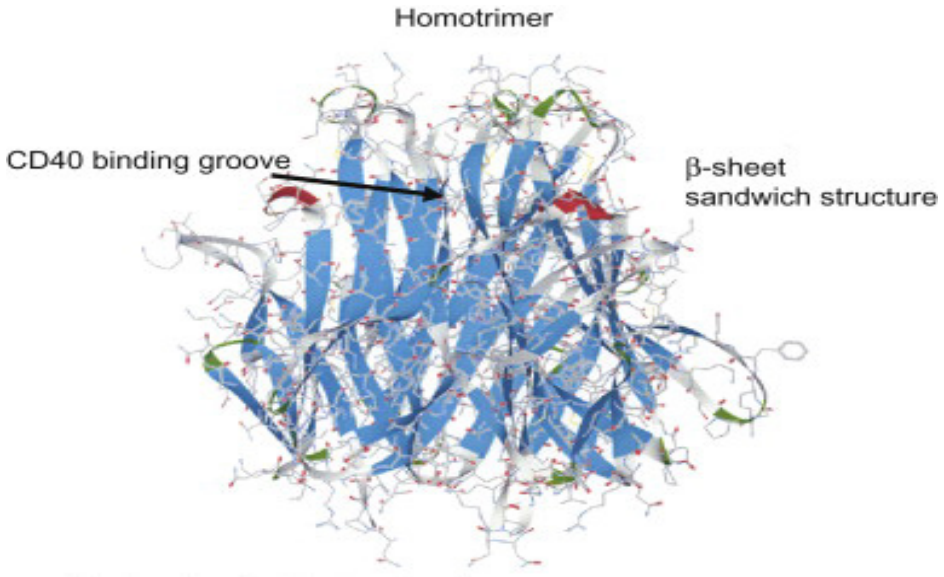

Figure 2.1: Modèle du complexe trimérique de CD40L. K. Clemenson et J.Clemenson. Chapitre : Platelet Receptors, p169. Platelets (Third Edition), AD. Michelson. *Academic Press* 2013

Cette structure faciliterait son interaction avec son récepteur principal, le CD40, afin de permettre l'induction de signaux intracellulaires. Cette interaction sera détaillée dans la section suivante. Outre sa forme membranaire, il existe la forme soluble de CD40L (sCD40L) provenant du clivage enzymatique du CD40L au niveau de la méthionine 113 de son domaine C terminal extracellulaire par des métalloprotéinases de matrice (MMPs) ou des ADAMs et maintient une forme trimérique fonctionnelle de 18 kDa (190). Le sCD40L est détecté dans la circulation sanguine; environ 95% du sCD40L provient de plaquettes, le reste des 5% sCD40L provient

surtout des lymphocytes. Le CD40L est exprimé constitutivement sur plusieurs types de cellules telles que les lymphocytes B, les cellules endothéliales, les neutrophiles et les macrophages. Cette expression du CD40L peut augmenter sur la surface de certaines de ces cellules suite à leur activation par des médiateurs inflammatoires comme les interleukines, l'interféron-γ ou TNF-α. Cependant, les plaquettes et les lymphocytes T n'expriment le CD40L que lorsqu'elles sont activées.

Le CD40L interagit avec les cellules du système vasculaire à travers quatre récepteurs. Pendant longtemps, le récepteur CD40 était considéré comme étant l'unique récepteur de CD40L; il aura fallu attendre l'année 2002 pour observer l'émergence d'autres récepteurs de CD40L, soit les intégrines $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ et $\alpha_M\beta_2$ (Mac-1) (191-193). La conformation trimérique du CD40L permet sa liaison à plusieurs récepteurs de même type comme les monomères de CD40 (on parle de liaison homotrimérique), mais aussi cette conformation lui confère la capacité de lier simultanément différents types de récepteurs due à la différence des résidus impliqués au niveau de la liaison du CD40L à chaque type de récepteurs (on parle de liaison hétérotrimérique) (194). Ainsi, la liaison du CD40L sur chacun des quatre récepteurs est bidirectionnelle, car elle implique l'activation des cellules exprimant le CD40L et les cellules interagissant avec le CD40L *via* l'expression des récepteurs aux CD40L. Cette interaction joue un rôle primordial dans la physiopathologie vasculaire associée à différentes maladies dont l'athéro-thrombose (Figure 2.2).

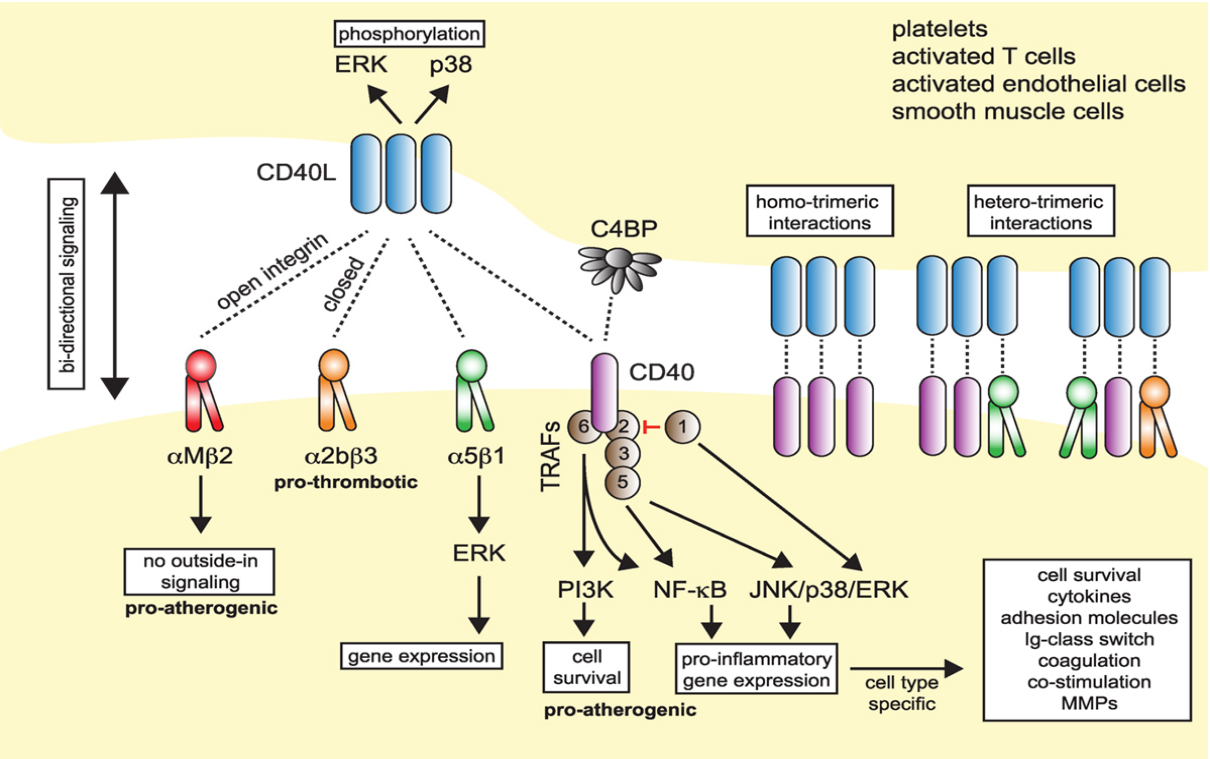

Figure 2.2: L'interaction du CD40L à ses récepteurs. Michel Na et al. *Front Cardiovasc Med.* 2017 Jun 20;4:40.

2.2.1 CD40

2.2.1.1 Structure et signalisation du CD40

Le CD40 est réputé pour être le récepteur classique du CD40L dû aux panoplies de fonctions qu'occupe l'axe CD40L/CD40 au sein de la physiopathologie. Aussi, il présente la plus grande affinité avec le CD40L (195-198). Le CD40 est une protéine transmembranaire de type I appartenant à la superfamille des récepteurs TNF de 48 kDa. Il a été d'abord identifié au niveau des lymphocytes B, où l'interaction CD40L/CD40 joue un rôle crucial dans leur prolifération, leur différentiation et leur capacité de production des anticorps. D'ailleurs, la première observation de la fonction du récepteur CD40 a été faite chez les patients souffrant du syndrome HIGM où l'absence de l'interaction CD40L avec les récepteurs CD40 exprimés sur les lymphocytes B entraine un défaut de la commutation isotypique du IgM. De plus, le complexe CD40L/CD40 est un médiateur important pour la prolifération des lymphocytes T et ses fonctions effectrices (187, 199-201). Une ambiguïté existe en ce qui concerne l'arrangement

conformationnel du CD40 sur la membrane des cellules; des études suggèrent que le CD40 est un trimère constitutivement associé en sa forme active sur la surface membranaire. Or, d'autres présentent le CD40 en tant qu'un assemblement de monomères ou de dimères qui se trimérisent sur le radeau lipidique suite à la liaison du CD40L (202-205).

L'interaction du CD40L au récepteur CD40 se fait grâce à la liaison polaire impliquant plusieurs résidus de CD40L et CD40 y compris les résidus basiques du CD40L comme K143, Y145, Y146 et R207 et les résidus acides du CD40 comme Y82, D84, E74 et E117. De plus, la liaison CD40L/CD40 est renforcée par un mur de résidus hydrophobique qui entoure l'interaction de ces deux groupes de résidus (206, 207). Par la suite, la liaison du CD40L au CD40 entraine le recrutement de protéines adaptatrices, nommées TRAF *(TNF receptor associated factor)* à la queue cytoplasmique de la molécule du CD40 (208). La famille des TRAFs rassemble six membres, TRAF1 à TRAF6. Ils sont composés d'un domaine C-terminal arrangés en sandwich β (TRAF-C ou MATH) permettant le recrutement des TRAFs sur la queue cytoplasmique du CD40, un domaine enroulé riche en leucine-zipper (TRAF-N) impliqué dans l'oligomérisation entre les TRAFs. Ce domaine est suivi par des motifs à doigts de zinc (à l'exception de TRAF1) qui permettent aux TRAFs d'agir en tant qu'ubiquitines ligases E3 (209, 210), afin de déclencher l'activation de plusieurs voies de signalisations en aval du CD40. On parle principalement de la voie du facteur NF-κB, les voies MAPKs dont p38, Akt, ERK1/2 et JNK ainsi que la voie PLCγ (208, 209). Cependant, d'autres voies peuvent être indépendantes du TRAFs; c'est le cas de la voie STAT5 qui découle de l'association directe de JAK3 au CD40 (211).

2.2.1.2 L'expression et fonction du CD40 sur les cellules

Le CD40 est constitutivement exprimé par les cellules présentatrices d'antigènes (CPA) comme les cellules dendritiques, les macrophages et les lymphocytes B et autres types de cellules. Cette expression augmente en présence de médiateurs pro-inflammatoires tels que TNF-α, IL-1, IFN-γ, CD40L (212, 213). La liaison du CD40L, exprimé par exemple sur les lymphocytes T au CD40 des CPAs, permet leur prolifération et leur différenciation, tels que la différenciation des lymphocytes B en lymphocytes B mémoires et en plasmocytes capables de produire des anticorps (187, 214). D'ailleurs, cette interaction permet la commutation isotypique permettant la production des IgM en IgG IgA et IgE (le IgE requiert la présence d'un signal

costimulateur comme IL-4 et IL-13) (215, 216) et la différentiation des monocytes en macrophages (217). De plus, cette liaison permet au CPAs la relâche des cytokines et chimiokines (IL-1, IL-6, IL-8, TNF-α, et autres) et de MMPs (MMP-1, MMP-2, MMP3, MMP-9 et autres) et l'expression de molécules costimulatrices (ICAM-1, LFA-1, B7.1, B7.2, CMH et autres), des médiateurs essentiels aux fonctions immunitaires, inflammatoires et angiogénique des CPAs (218-221). Les CPAs jouent aussi un rôle primordial dans l'activation des lymphocytes T; en effet, l'interaction du CD40 des CPAs avec le CD40L des lymphocytes T est bidirectionnelle, de sorte que l'activation des CPAs par le CD40L des lymphocytes T permet à son tour l'activation des lymphocytes T par le CD40 des CPAs et leur récepteur CMH (complexe majeur d'histocompatibilité). Effectivement, l'activation des lymphocytes T sollicite deux signaux de la part des CPAs; le premier est suite à l'interaction du CMH des CPAs qui présentent un antigène spécifique au récepteur des cellules T (TCR) des lymphocytes T. Ce signal induit l'expression à la surface des lymphocytes T des récepteurs costimulatrices CD28 et CD40L, qui se lient respectivement avec les récepteurs B7.1 ou B7.2 et CD40 des CPAs. Ainsi, ce signal d'activation bidirectionnelle CD40L/CD40 induit la prolifération et la différenciation des lymphocytes T en des lymphocytes T effecteurs et parallèlement, permet la différentiation des CPAs tels que les lymphocytes B en plasmocytes et lymphocytes B à mémoire, des monocytes en macrophages et amplifie l'expression membranaire des molécules costimulatrices et CMH des cellules dendritiques et la relâche de l'IL-12 qui, en combinaison avec le CD40L, induit la différenciation des lymphocytes T en lymphocytes T auxiliaires Th1 (187, 222, 223). Ces interactions sont au cœur des réactions immunitaires et inflammatoires. De plus, le CD40L exprimé à la surface des lymphocytes T, suite à leur activation, est clivé par l'ADAM10 et ADAM17, et libéré sous forme de sCD40L, représentant 5% du sCD40L présent en circulation (95% du sCD40L provenant des plaquettes)(224, 225).

Hormis les CPAs et les lymphocytes T, le CD40 est exprimé constitutivement sur les cellules endothéliales, les cellules musculaires lisses (CML), les neutrophiles et les plaquettes. La liaison du CD40L avec le CD40 des cellules endothéliales joue un rôle important dans la réaction inflammatoire. Suite à l'activation de l'endothélium *via* le CD40 suite au recrutement des leucocytes et des plaquettes activées qui expriment à leur surface le CD40L, les cellules endothéliales déploient une panoplie de protéines qui amplifie l'inflammation au site de leur activation par leur capacité à exprimer à leur surface des récepteurs d'adhésion tels que les

ICAM-1, VCAM-1, les P-séléctines et E-séléctines ainsi que la sécrétion de cytokines et chimiokines dont IL-1, IL-6, IL-8, MCP-1, MIP-1α et RANTES (226-228). Dès lors, l'activation du CD40 au niveau de l'endothélium participe à l'initiation des pathologies à caractère inflammatoire, dont l'athérosclérose qu'on discutera dans la prochaine section. D'ailleurs, l'activation du CD40 des cellules endothéliales contribue à l'angiogenèse *via* le relâche des MMPs (MMP-1, MMP2- et MMP-9) et des facteurs de croissance comme la VEGF et bFGF favorisant la progression de l'athérosclérose (229, 230). Enfin, comme on l'a mentionné dans la section 1.4.1 sur la thrombose, les cellules endothéliales expriment à leur surface TF qui est aussi médiée par l'interaction du CD40L sur les récepteurs CD40 de l'endothélium activé, induisant la cascade de coagulation et l'activation plaquettaire (231, 232).

La présence des molécules pro-inflammatoires sécrétées par les leucocytes et plaquettes au site de l'endothélium activé dont le TNF-α, IL-1β, et IFN-γ entraine une augmentation de l'expression de CD40 sur les CML. L'interaction de CD40L avec le CD40 des CML provoque une réaction similaire aux cellules endothéliales, et cela par l'expression de récepteurs d'adhésions (ICAM-1, VCAM-1, E-séléctine) et sécrétion de cytokines, chimiokines et MMPs favorisant leur prolifération et migration au site de l'endothélium activé afin de participer à la formation et le développement des plaques d'athérome (233-236). De plus, hormis ces fonctions proatherogénique, elles ont des fonctions pro-thrombotiques en recrutant les plaquettes et par sa capacité à exprimer le TF (237).

Le CD40 est aussi présent sur les neutrophiles; ces derniers sont les plus abondants leucocytes en circulation et sont les premiers intervenants au site d'inflammation. L'interaction du CD40L avec le CD40 des neutrophiles est majeure dans les interactions avec les plaquettes; en effet, la liaison du CD40L plaquettaire (membranaires et solubles) avec le CD40 des neutrophiles entraine leur activation et la sécrétion des DRO (Dérivés réactifs de l'oxygène). Les DRO générés par les neutrophiles provoquent réciproquement l'activation de plaquettes additionnelles, créant une sorte de rétroaction positive d'activation entre plaquettes et neutrophiles (238). La présence du CD40L plaquettaire favorise la surexpression du CD40 et Mac-1 et incite l'apparition d'agrégats plaquettes/neutrophiles, de même que la formation néointimale en un site de lésion vasculaire (239, 240). Enfin le CD40 est constitutivement sur les plaquettes et la voie CD40L/CD40 plaquettaire joue un rôle primordial dans les pathologies

thrombo-inflammatoires, l'interaction du CD40 plaquettaire avec l'endothélium activé, monocytes et leucocytes exprimant le CD40L permet aux plaquettes de provoquer une réaction inflammatoire majeure par leur formidable répertoire en cytokines, chimiokines, facteurs de croissance et angiogéniques. L'axe CD40L/CD40 plaquettaire sera discuté davantage dans la section 2.3.

2.2.2 $\alpha_{IIb}\beta_3$

Comme on l'a mentionné dans le chapitre des plaquettes, l'$\alpha_{IIb}\beta_3$ est l'intégrine la plus exprimée sur les plaquettes et elle est primordiale à la fonction plaquettaire et surtout au phénomène de l'agrégation en liant le fibrinogène. Ainsi, cette intégrine lie le fibrinogène grâce au motif RGD. Cependant, la découverte de la capacité du poison de vipère (Sistrurus miliarius barbouri) à interagir avec un motif KGD (Lysine-Glycine-Acide aspartique) de l'$\alpha_{IIb}\beta_3$ a permis l'émergence de la possibilité que le CD40L puisse lier l'$\alpha_{IIb}\beta_3$ des plaquettes *via* ce motif (241). En effet, en 2002, il a été démontré que le CD40L interagit avec l'$\alpha_{IIb}\beta_3$ *via* le motif KGD et induit sur des plaquettes activées leur étalement, favorise l'agrégation plaquettaire dans des conditions de cisaillement élevé ainsi que la stabilisation des thrombi artériels in vivo (191) (Figure 2.3). Cette interaction engendre l'activation plaquettaire par une signalisation «*outside-in*» suite à la phosphorylation des tyrosines présentes sur la queue cytoplasmique β_3 de l'intégrine (242). Enfin, la liaison du CD40L avec l'$\alpha_{IIb}\beta_3$ favorise l'adhésion plaquettaire sur l'endothélium et participe à la surexpression du CD40L sur les cellules endothéliales.

2.2.3 $\alpha_5\beta_1$

Comme discuté dans la section 1.2.2.3, l'intégrine $\alpha_5\beta_1$ est le récepteur plaquettaire principal de la fibronectine qu'elle lie *via* la séquence RGD. Hormis les plaquettes, il est exprimé sur différentes cellules, dont les monocytes/macrophages, les cellules endothéliales et les cellules stromales (192). L'$\alpha_5\beta_1$ joue un rôle important dans l'adhésion, la migration, la survie et la prolifération cellulaire; elle participe aussi à l'angiogenèse par sa capacité à interagir avec le VEGFR-1, l'angiopoietine-2 et l'endostatine (243-246). L'$\alpha_5\beta_1$ a été identifié comme étant un récepteur de CD40L sur la ligné monocytaire U937 et la liaison du CD40L à l'intégrine induit une activation en aval de $\alpha_5\beta_1$ similaire à celle induite par la fibronectine, soit par l'activation de la MAPK, ERK1/2 et l'expression génique de l'IL-8. De plus, le CD40L ne peut interagir

qu'avec la forme inactive de l'$\alpha_5\beta_1$ (192). Récemment, il été démontré que la liaison du CD40L avec l'$\alpha_5\beta_1$ entraine la génération des cytokines et l'adhésion du CD40L des lymphocytes T avec les fibroblastes (247); aussi, elle bloque l'apoptose dans les lymphocytes T (248). Enfin, une étude suggère que l'inhibition de $\alpha_5\beta_1$ atténue l'activation plaquettaire entrainée par le CD40L (249).

2.2.4 $\alpha_M\beta_2$

L'intégrine $\alpha_M\beta_2$ ou Mac-1 est composé de deux sous-unités, α_M et β_2. Elle est exprimée sur différents leucocytes impliqués dans l'immunité dont les monocytes/macrophages, neutrophiles et les cellules NK (Natural Killers). Le Mac-1 participe au roulement et l'adhésion des leucocytes sur l'endothélium activé (250, 251), et cela par sa capacité à lier une panoplie de ligands impliqués dans le recrutement des leucocytes comme ICAM-1, le fibrinogène, la fibronectine, le GPIb-IX-V (251-254). Le Mac-1 a été identifié comme étant un récepteur du CD40L sur des monocytes activés par le PMA (phorbo-12-myristate-13-acetate) en liant le domaine I de la sous unité α_M; la liaison de haute affinité entre le CD40L et Mac-1 requiert le changement conformationnel de l'intégrine suite à son activation (255, 256). L'interaction entre le CD40L et Mac-1 engendre l'adhésion et la migration des monocytes au sein de site de formation de la plaque d'athérome menant à la sécrétion des cytokines, chimiokines et myélopéroxydases favorisant la réaction inflammatoire et la progression athérosclérotique. Par conséquent, l'inhibition de Mac-1 réduit l'athérosclérose chez les souris (255). De plus, les cellules endothéliales exprimant CD40L recrutent les macrophages, exprimant hautement le Mac-1, et participent à leur activation, l'expression des gènes inflammatoires et contribuent à leur fonction phagocytaire (257-259). Ainsi, l'interaction CD40L/Mac-1 joue un rôle primordial dans différents processus physiopathologiques dont l'inflammation et la réaction immunitaire (258).

2.3 Le CD40L plaquettaire

En 1998, Hen et al. découvrent que les plaquettes activées expriment le CD40L à leur surface *in vitro* et suite à la formation du thrombus *in vivo*. Cette étude identifie chez les plaquettes une fonction au-delà de leur rôle classique au niveau de l'hémostase. En effet, le

CD40L plaquettaire était capable d'induire la sécrétion de chimiokines et de molécules d'adhésion par les cellules endothéliales et de promouvoir le recrutement des leucocytes au site de lésion (260). Ainsi, la découverte du CD40L sur les plaquettes est l'un des piliers fondateurs dans l'émergence des études qui exploreront l'implication des plaquettes dans les réactions inflammatoires dont l'athérosclérose et les pathologies immunitaires.

2.3.1 Expression du CD40L plaquettaire

Les plaquettes sont dépourvues de CD40L à leurs surfaces membranaires quand elles sont au repos. Or, suite à leur activation par des agonistes plaquettaires (thrombine, collagène ou ADP), le CD40L transloque à la membrane des plaquettes. Grâce à la technique d'immunocoloration en microscopie, le CD40L a été identifié comme étant stocké dans les α-granules au sein du cytosol (261). De même, les patients souffrants du syndrome des plaquettes grises se caractérisent par des plaquettes qui ne contiennent pas d'α-granules et n'expriment pas de CD40L (261). Le stockage du CD40L semble être réalisé au sein des mégacaryocytes avant la formation des plaquettes. En effet, les mégacaryocytes possèdent l'ARNm (ARN messager) du CD40L, son expression augmente suite à la différentiation des mégacaryocytes (262). De plus, malgré que les plaquettes possèdent la machinerie pour produire des protéines de novo comme des cytokines, cela ne paraît pas être le cas pour le CD40L. Justement, le séquençage d'ARNm au sein des plaquettes ne montre pas la présence d'ARNm de CD40L (263-266).

La structure du CD40L plaquettaire exprimé demeure sous la forme d'un trimère associé par des liens non covalents (267). Suite à l'activation plaquettaire et la dégranulation des α-granules, l'expression du CD40L à la membrane ne dure que quelques minutes à une heure. Effectivement, il est ensuite clivé de la membrane plasmatique en un fragment extracellulaire trimérique, le sCD40L de 18 kDa. Il est important de mentionner que 95% du sCD40L est d'origine plaquettaire, le 5% provient des lymphocytes T activés (222, 260). La concentration moyenne du sCD40L sérique d'un adulte est estimée entre 0,79 et 4,7 ng/ml; cependant, chez un patient diabétique ou thrombotique, elle peut atteindre 50 ng/ml (268-270). Le mécanisme impliquant la conversion du CD40L en sCD40L demeure intrigant, mais différentes études essayent d'élucider le mécanisme exact du clivage du CD40L plaquettaire. L'activité protéolytique des MMPs serait fortement impliquée dans le clivage du CD40L plaquettaire, plus précisément la MMP-2 et la MMP-9 plaquettaire (271, 272) à l'opposé du clivage du CD40L

des lymphocytes T activé qui implique ADAM10 et ADAM17 (224, 225). Le clivage du CD40L est $\alpha_{IIb}\beta_3$-dépendant; en effet, les antagonistes $\alpha_{IIb}\beta_3$ (abciximab ou Reopro) empêchent le relâche du sCD40L en circulation. De plus, les patients souffrant du syndrome de Glanzmann présentent beaucoup moins le sCD40L au sein de leur circulation sanguine (273, 274). Un mécanisme nécessitant l'interaction entre l'$\alpha_{IIb}\beta_3$ et MMP-2 pourrait être impliqué (275). D'ailleurs, il a été démontré que l'activation du NADPH (Nicotinamide adenine dinucleotide phosphate) et la génération des DRO par les plaquettes activées ainsi que la liaison du CD40L au CD40 contribuent au clivage du CD40L membranaire (276, 277). De plus, le sCD40L pouvant participer à l'activation plaquettaire suggère une certaine une rétroaction positive amplifiant la sécrétion du sCD40L (242, 278). Le CD40L peut aussi prendre une autre forme soluble, soit en s'attachant sur les MP. Effectivement, les MP sont relâchés suite à l'activation plaquettaire et une forme fonctionnelle du CD40L est exprimée sur ces MP (279-281). Malgré les défis que rencontrent les méthodes d'ELISA à identifier le sCD40L du CD40L lié aux MP, les CD40L liés aux MP pourraient jouer un rôle dans l'amplification des réactions inflammatoires (282). Enfin, le taux de sCD40L en circulation corrèle avec l'activation des plaquettes et représente ainsi un biomarqueur important puisqu'il sollicite des réponses cellulaires thrombo-inflammatoires (283-286). D'ailleurs le taux élevé de sCD40L en circulation est considéré comme un prédicteur fiable des maladies cardiovasculaires (267), surtout chez les patients souffrant de diabète (269, 287, 288), d'hypercholestérolémie (289, 290), d'athérosclérose (291) et de SCA (270, 292, 293).

2.3.2 La signalisation des récepteurs du CD40L sur les plaquettes

Comme on l'a mentionné auparavant, il y a quatre récepteurs qui peuvent s'associer avec CD40L, soit CD40, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, Mac-1. Les plaquettes n'expriment pas Mac-1; ainsi, elles possèdent trois récepteurs de CD40L, soit CD40, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$.

Les plaquettes expriment constitutivement le CD40, la liaison CD40L/CD40 induit l'activation des plaquettes en engendrant leur changement de forme, l'expression de la P-séléctine à la surface plaquettaire ainsi que la sécrétion des α-granules et granules dense. Malgré que cette liaison favorise une activation de l'intégrine $\alpha_{IIb}\beta_3$, elle est incapable de provoquer l'agrégation plaquettaire (260, 278). Le CD40L joue le rôle d'un amorceur ou «*primer*»; ainsi, il peut déclencher une agrégation plaquettaire en présence de doses sous-optimales d'agonistes

plaquettaires comme la thrombine et le collagène. Effectivement, au sein de notre laboratoire, on a trouvé que le CD40L amorce les plaquettes en activant la voie de signalisation CD40/TRAF2/Rac-1/p38 MAPK et induit l'agrégation plaquettaire en présence de doses sous-optimales d'agonistes. Les plaquettes traitées avec une forme de sCD40L mutée qui ne reconnait pas CD40 ou les plaquettes de souris déficiente en CD40 ($CD40^{-/-}$) sont incapables d'induire une telle réponse. De plus, un modèle de thrombose artérielle induite par lésion avec $FeCl_3$ démontre que l'infusion de sCD40L de souris exacerbe la formation de thrombose et l'infiltration leucocytaires chez les souris sauvages (WT), mais pas chez les souris $CD40^{-/-}$ (294). Une autre étude suggère que le sCD40L amorce les plaquettes et les agrège en présence de doses sous-optimales d'agonistes *via* une voie de signalisation activant NF-κB, une voie indépendante de p38 MAPK. Cette voie pourrait être activée par le récepteur CD40 aussi en se basant sur l'activation du NF-κB par le CD40 dans les cellules nucléées suite à la liaison au CD40L (295). La liaison du CD40L au CD40 plaquettaire induit la sécrétion de DRO et des chimiokines comme PF4, MCP-1 et RANTES (296-299). Ainsi, l'interaction CD40L/CD40 est fortement impliquée dans les phénomènes athéro-thrombotique en favorisant le recrutement leucocytaire, la formation des agrégats plaquettes-leucocytes, l'adhésion des plaquettes à l'endothélium activé et la progression de la plaque athérosclérotique (298, 299).

Comme on l'a déjà mentionné, la liaison du CD40L à l'$\alpha_{IIb}\beta_3$ induit l'activation plaquettaire et participe à l'agrégation plaquettaire dans des conditions de cisaillement élevé ainsi que la stabilisation des thrombi artériels in vivo (191). La liaison CD40L/$\alpha_{IIb}\beta_3$ engendrant l'étalement plaquettaire, la formation du thrombus, l'expression du CD40L et la génération des MP *via* la signalisation «*outside-in*» de l'intégrine permettent la phosphorylation de la tyrosine-759 du domaine cytoplasmique de la sous-unité β3 de l'intégrine. Cette signalisation déclenche par conséquent une boucle d'auto-amplification favorisant la stabilisation du thrombus (242, 267). Une étude suggère que le CD40L favorise l'activation et l'agrégation plaquettaire en présence de collagène ainsi que la formation du thrombus en liant l'$\alpha_{IIb}\beta_3$ en activant la voie PI3K (300).

Peu d'informations est connue sur la liaison du CD40L sur l'$\alpha_5\beta_1$; une étude propose que l'$\alpha_5\beta_1$ serait impliqué dans l'activation plaquettaire. En effet, l'incubation des plaquettes avec l'anticorps anti-$\alpha_5\beta_1$ réduit l'activation plaquettaire médiée par le sCD40L, diminuant

l'expression de la P-séléctine et PAC-1 (*Procaspase activating compound*), l'épitope d'activation de l'$\alpha_{IIb}\beta_3$ dans les plaquettes humaines (249). Ainsi, la capacité du CD40L à lier les récepteurs plaquettaires de CD40L de façon hétérodimérique suggère que l'action du $\alpha_5\beta_1$ sur l'activité des plaquettes serait en coopération avec les récepteurs CD40 et $\alpha_{IIb}\beta_3$ (194, 249).

2.4 CD40L et pathologies

Le CD40L joue une rôle physiologique important dont l'immunité cellulaire et humorale. En effet, le syndrome HIGM a permis l'émergence de l'importance du CD40L dans la réaction immunitaire où il joue un rôle primordial dans la prolifération et la différentiation des lymphocytes T et B ainsi que leur activation en interagissant avec les CPAs. Cependant, un manque de régulation de l'expression du CD40L ou des interactions atypiques du CD40L avec ces récepteurs peut engendrer une panoplie de pathologies dont les maladies auto-immunitaires, le cancer et l'athéro-thrombose.

2.4.1 Les maladies auto-immunitaires.

L'interaction CD40L/CD40 joue un rôle crucial dans les maladies auto-immunitaires en favorisant la production d'autoanticorps et une hyperactivité immunitaire ciblant l'organisme. Plusieurs mécanismes peuvent engendrer le développement des maladies auto-immunitaires, d'abord au niveau des organes lymphoïdes primaires, une hyperactivation ou un défaut d'activation du CD40 au niveau du thymus lors de la sélection des lymphocytes T peut entrainer l'évasion des lymphocytes T auto-réactifs de la sélection négative. Effectivement, les cellules épithéliales médullaires du thymus sont responsables aussi de la délétion des lymphocytes auto-réactifs *via* un mécanisme impliquant le récepteur CD40 et RANK (*Receptor activator of NF-κB*) (301). Ensuite, au niveau des organes lymphoïdes secondaires, une hyperactivation du CD40 des lymphocytes B et des cellules dendritiques causée par l'action d'une surexpression de CD40L des lymphocytes T pourrait promouvoir une sécrétion accrue de cytokines et d'auto-anticorps en circulation participant à l'occurrence des maladies auto-immunes (302). On retrouve d'ailleurs une surexpression du CD40L chez différents patients souffrant de maladies auto-immunes comme le lupus érythémateux disséminé, l'arthrite rhumatoïde, la sclérose en plaques, la maladie inflammatoire de l'intestin et la psoriasis (303-307). Enfin, on observe chez les patients atteints de maladies immunitaires une expression importante du CD40 au niveau des

tissus ou organes qui expriment normalement peu ou pas le CD40, favorisant une interaction plus importante avec le CD40L. Par exemple, on retrouve une expression membranaire accrue de CD40 sur les cellules de la glande thyroïde dans la maladie de Graves et du pancréas dans le diabète de type 1. D'ailleurs, la surexpression du CD40 induit la génération de cytokines qui participent au rejet de transplantations d'ilots pancréatiques chez les patients souffrant de diabète (308-311).

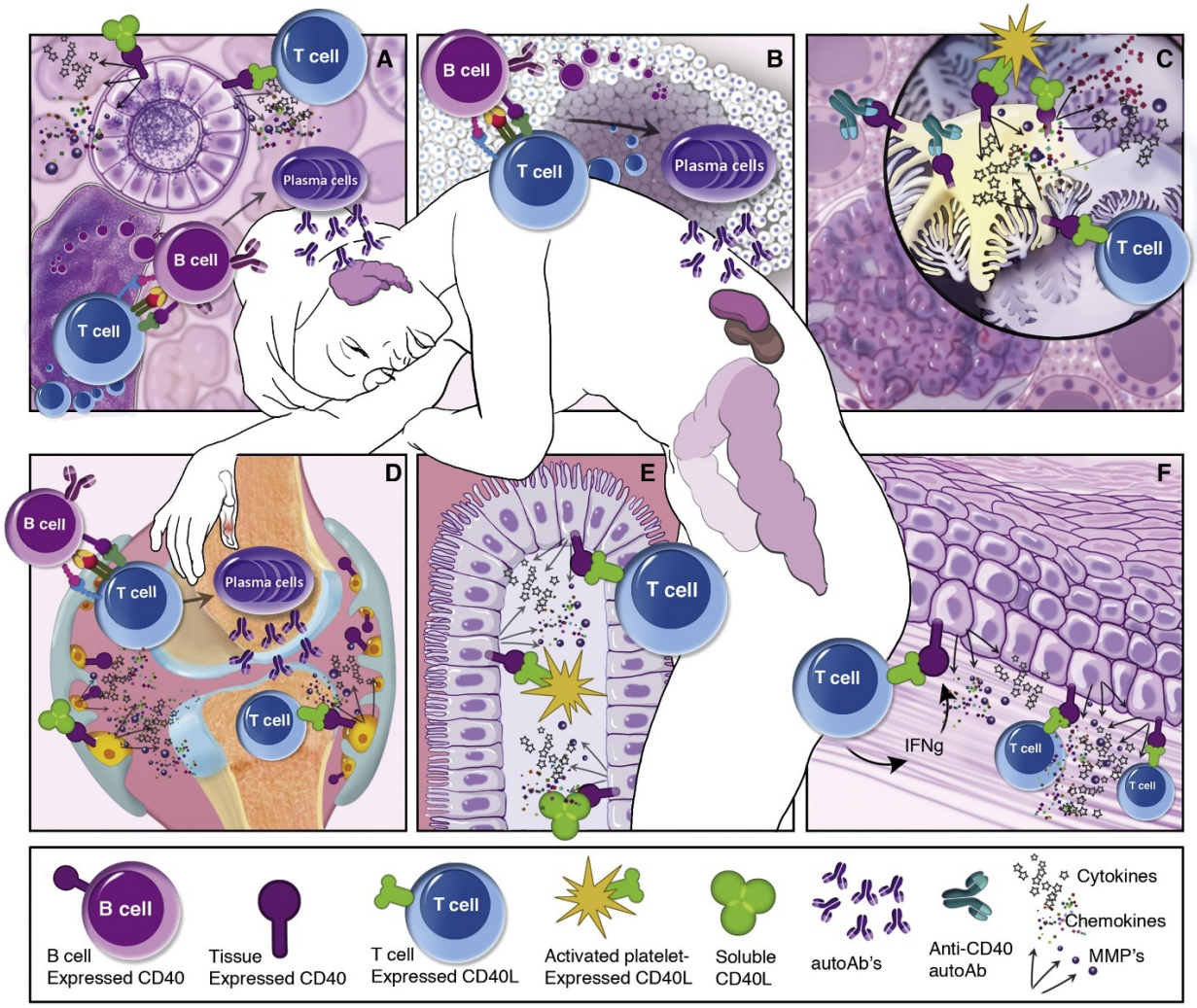

Figure 2.3: La contribution du CD40L dans les pathologies auto-immunitaires. Karnel JL et al. *Adv Drug Deliv Rev*. 2018 Dec 13. pii: S0169-409X(18)30308-9

L'interaction CD40L/CD40 sont au centre de différents organes qui peuvent développer des maladies auto-immunitaires dont les glandes salivaires dans le syndrome de Sjögren. En

effet, le CD40 exprimé par les cellules épithéliales de glandes salivaires lie le CD40L des lymphocytes T et le sCD40L des plaquettes activées provoquant la sécrétion de cytokines, chimiokines et MMPs. De plus, le CD40 des lymphocytes B interagissant avec le CD40L des lymphocytes T peut se différencier en des plasmocytes autoréactifs (Figure 2.3 A). Au niveau de la rate, les lymphocytes T du centre germinal contribuent à la différenciation plasmocytaire et la commutation des lymphocytes B du tissu lymphoïde (Figure 2.3 B). Au niveau des reins, l'interaction CD40L/CD40 contribue aux néphropathies auto-immunitaires - par exemple, la liaison du CD40 exprimé par les podocytes de la capsule de Bowman au CD40L des lymphocytes T, du CD40L membranaire et soluble des plaquettes activées ou des auto-anticorps anti-CD40 agonistes qui sont produits lors de la glomérulosclérose segmentaire et focale peuvent contribuer à la pathogenèse de la néphropathie auto-immunitaire en altérant la perméabilité glomérulaire (Figure 2.3 C). Au niveau des articulations, l'expression du CD40 sur les synoviocytes peut contribuer à l'arthrite rhumatoïde à la suite de leur interaction avec le CD40L des lymphocytes T et CD40L solubles des plaquettes activées, en entrainant la destruction de l'articulation, en déclenchant la production de médiateurs proinflammatoires. De plus, des lymphocytes B exprimant CD40 activées par le CD40L des lymphocytes T peuvent produire localement des auto-anticorps (Figure 2.3 D). Au niveau de l'intestin, la voie CD40L/CD40 est impliquée dans les maladies inflammatoires de l'intestin. En effet, les cellules épithéliales de l'intestin de la muqueuse enflammée expriment fortement le CD40; ainsi, ces cellules secrètent cytokines, chimiokines et MMPs qui favorisent le recrutement des lymphocytes, neutrophiles et plaquettes activées qui expriment le CD40L. Cela mène à l'amplification de la réaction inflammatoire (Figure 2.3 E). Enfin, au niveau cutané, le CD40 est exprimé de façon importante sur les kératinocytes dans des maladies telles que le psoriasis et le lupus érythémateux systémique. L'interaction du CD40L des lymphocytes T infiltrant le milieu épidermique contribue à la production de cytokines et chimiokines pouvant provoquer la progression des maladies auto-immunitaires cutanées (Figure 2.3 F)(221).

2.4.2 Le cancer

La littérature suggère un rôle paradoxal du CD40L dans le cancer. Effectivement, les études sur l'implication du CD40L dans le cancer sont controversées et ainsi, on observe que le CD40L pourrait à la fois induire le développement du cancer, comme il peut la réduire. Ce qui

semble expliquer cette fonction paradoxale du CD40L dans la pathogenèse du cancer est l'expression du CD40 sur la surface des cellules cancéreuses qui peut varier entre les types de cellules néoplasiques (Figure 2.4) (312)

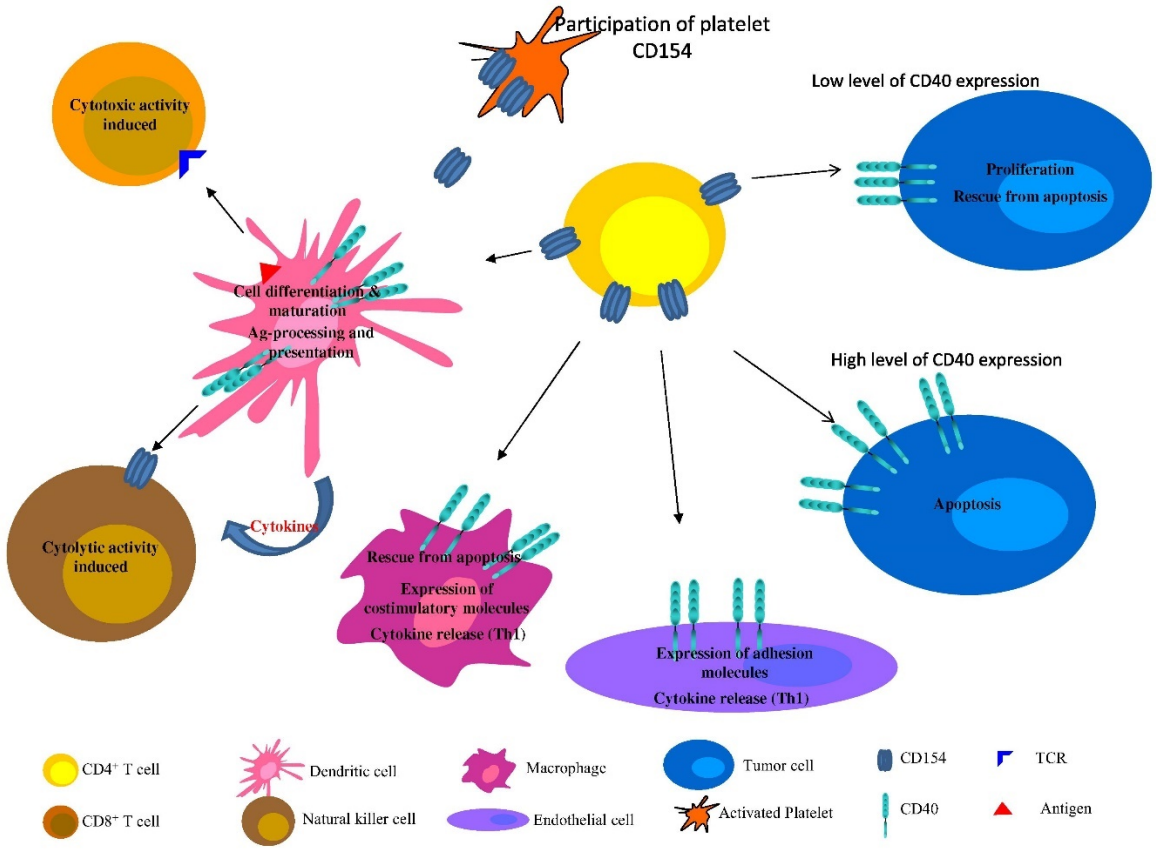

Figure 2.4: Rôle du CD40L dans le développement et l'élimination du cancer. Hassan GS et al. *Cancer Treat Rev*. 2015 May;41(5):431-40.

Ainsi, le CD40 est exprimé sur plusieurs types de tumeurs; d'ailleurs, le CD40 a d'abord été identifié sur les cellules du lymphome de Burkitt (201, 313). Suite à cela, des études ont démontré sa présence sur de multiples tumeurs dont le mélanome, l'ostéosarcome, lymphome non-hodgkinien et plusieurs types de carcinomes (314-319). Dès lors, l'expression répandue du CD40 sur les cellules tumorales implique que l'interaction CD40L/CD40 joue un rôle dans la pathogenèse du cancer. Effectivement, des études démontrent qu'une faible expression constitutive de CD40 par les cellules néoplasiques interagissant avec le CD40L peut contribuer

à leur croissance. En effet, une faible génération de CD40L par les cellules tumorales comme ceux du lymphome non-hodgkinien, lymphome de Burkitt et le cancer du sein est capable de promouvoir une prolifération maintenue et une protection de l'apoptose chez ces cellules par un mécanisme impliquant le NF-κB et ainsi favoriser la progression tumorale (320-322). On observe chez les cellules tumorales qui co-expriment faiblement le CD40L et le CD40, un mécanisme autocrine qui amplifie la croissance des lymphomes et carcinomes. De plus, les lymphocytes T exprimant le CD40L participent à la survie cellulaire des cellules cancéreuses notamment dans la leucémie lymphoïde chronique, en activant le NF-κB. Enfin, l'utilisation d'anticorps anti-CD40L, afin de bloquer l'interaction CD40L/CD40 au niveau des cellules qui expriment faiblement CD40L et CD40, est capable d'inhiber l'activation du NF-κB et, par conséquent, prévient la croissance tumorale et active l'apoptose cellulaire (320-323).

En revanche, d'autres études démontrent que l'axe CD40L/CD40 joue plutôt un rôle négatif dans la pathogenèse tumorale. Un des cas qui soutient cet argument est la fréquence élevée de tumeurs chez les patients atteints du HIGM. Ces patients, comme on l'a mentionné auparavant, n'expriment pas le CD40L (324). Dans des modèles murins, des souris déficientes en CD40L perdent leur mécanisme anti-tumoral immunitaire contre différents types de tumeurs dont l'adénocarcinome, le carcinome de la vessie, mélanome et fibrosarcome (325, 326). De plus, le traitement de souris atteints de tumeurs avec un anticorps CD40 agoniste induit une diminution de la prolifération tumorale due à une augmentation de l'activité des lymphocytes T cytotoxiques et des cellules NK (327, 328). D'ailleurs, les cellules cancéreuses des tumeurs comme la leucémie lymphoïde chronique altèrent l'interaction CD40L/CD40 en clivant le CD40L ou en empêchant l'expression du CD40L membranaire des lymphocytes T afin d'échapper le mécanisme anti-tumoral des lymphocytes T cytotoxiques et des cellules NK (329). De plus, le CD40 subit un clivage enzymatique ou une mutation par les cellules tumorales afin de dévier l'interaction du CD40L. Ainsi, on retrouve un niveau élevé de CD40 soluble en circulation chez les patients atteints, par exemple, de leucémie myéloïde aigüe et du myélome multiple (330) ou une diminution d'expression du CD40 comme chez les patients atteints de lymphome non hodgkinien (353). Comme on l'a mentionné au début, l'expression de CD40 peut moduler la fonction du CD40L. Ainsi, une expression élevée du CD40 par les cellules tumorales induit la fonction anti-tumorale du CD40L. Dès lors, hormis l'activité des lymphocytes T cytotoxiques et des cellules NK médiant l'éradication de la tumeur, l'activation

du CD40 des cellules tumorales engendre la production des médiateurs proapoptotiques comme FasL (*Fas ligand*), TNF et TRAIL (*TNF-related apoptosis-inducing ligand*) induisant l'apoptose des cellules tumorales de façon paracrine ou autocrine (331-333). De plus, la liaison du CD40 entraine l'expression de molécules d'adhésion comme le TAP1 (*Antigen peptide transporter-1*), permettant alors la reconnaissance des cellules tumorales par les cellules immunitaires anti-tumorales (334, 335).

2.4.3 L'athérosclérose

L'athérosclérose est une maladie inflammatoire chronique qui se caractérise par l'accumulation d'une plaque de dépôt lipidique dans la couche interne ou intima d'un vaisseau sanguin, entrainant ainsi une diminution du flux sanguin et l'occurrence d'une occlusion thrombotique. Le développement de la plaque riche en lipides comme le LDL et l'activation de l'endothélium incitent le recrutement de cellules qui médient l'inflammation dont les leucocytes, les CML et les plaquettes. L'activation de ces cellules vasculaires crée un milieu riche en médiateurs pro-inflammatoires et pro-athérogéniques comme les cytokines, chimiokines et MMPs. L'expression répandue du CD40L sur les cellules qui régissent l'athérogénèse suggère une contribution importante du CD40L au sein de l'athérosclérose (336) (Figure 2.5).

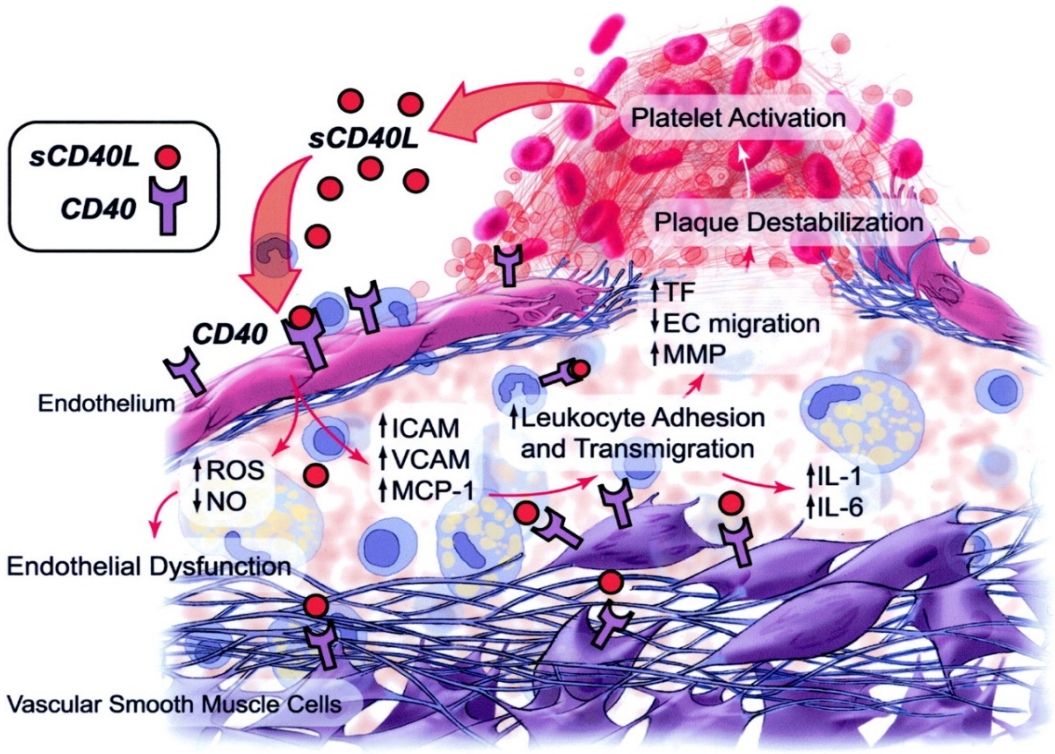

Figure 2.5: L'interaction CD40L/CD40 dans l'athérosclérose. Szmitko PE et al. *Circulation.* 2003 Oct 21;108(16):1917-23.

2.4.3.1 L'initiation de la plaque

L'initiation de la plaque d'athérosclérose se caractérise par l'activation de l'endothélium suite à l'accumulation de oxLDL et le recrutement des lymphocytes T et des monocytes. Ainsi, les oxLDL engendrent une surexpression du CD40 et CD40L des cellules endothéliales *via* l'activation du PKCα (337, 338) . De plus, les cellules immunocompétentes, dont les lymphocytes T, amplifient l'expression du CD40 sur les membranes des cellules endothéliales suite à leur sécrétion de cytokines comme IL-1, TNF-α, IFN-γ ainsi que par l'interaction du CD40L exprimé sur les lymphocytes T avec le CD40 des cellules endothéliales *via* l'activation du NF-κB (339-341). En outre, la liaison du CD40 des cellules endothéliales avec le CD40L des cellules immunocompétentes et des plaquettes activées induit la sécrétion de médiateurs pro-inflammatoires et pro-athérogéniques dont des molécules d'adhésion comme P-séléctine, E-séléctine, ICAM-1 et VCAM-1, une panoplie des cytokines et chimiokines comme IL-6, IL-8, IL-15, MCP-1, MIP-1α et RANTES (342-345). Ainsi, cela favorise la transmigration de leucocytes à l'endothélium activé. L'interaction CD40L/CD40 au niveau des

cellules endothéliales entraine une expression accrue de DRO et une diminution d'ARNm responsable de l'expression de l'oxyde nitrique synthase (NOS) et déstabilise son activité enzymatique, ce qui entraine une réduction des mécanismes antiinflammatoires de l'endothélium (346). Le CD40L promeut aussi la formation d'agrégats plaquettes-leucocytes qui amplifie le recrutement leucocytaire au niveau de l'endothélium *via* les récepteurs CD40 et Mac-1 (239, 298).

2.4.3.2 La progression de l'athérosclérose

Plusieurs évènements régissent la progression de l'athérosclérose. D'abord, la transmigration des monocytes engendre leur différentiation en macrophages capable de phagocyter l'oxLDL de l'espace sous-endothéliale. Ces macrophages se développent en des cellules spumeuses. Ces cellules spumeuses exprimant le CD40 interagissent avec les lymphocytes T infiltrés, ce qui provoque la production de molécules favorisant la réponse athérogénique dont les cytokines (IL-1β, IL-6, IL-12, et TNFα), MMPs (MMP-1 et MMP-3) et facteur de croissance (VEGF) (212, 336, 347). De plus, ces macrophages modifiés sont capables d'amplifier leur activation de façon autocrine en exprimant le CD40L (344). Dès lors, les réactions pro-athérogéniques et pro-inflammatoires induites par les cellules spumeuses jouent un rôle important dans la prolifération des CML et leur migration au niveau de l'intima du vaisseau, un événement clé dans la progression de l'athérosclérose (235). En effet, la liaison du CD40L au CD40 des CML entraine la sécrétion de médiateurs contribuant à l'athérogénèse dont des cytokines incluant IL-1β, IL-6, IL-8 et MCP-1 ainsi que des MMPs comme MMP9 (213, 233, 235). Parallèlement, le CD40L engendre la prolifération des fibroblastes ainsi que l'expression de molécules d'adhésion et la génération de cytokines pro-inflammatoires, participant ainsi à l'exacerbation de l'athérosclérose (348, 349). Enfin, la capacité du CD40L à entrainer la génération de facteurs pro-angiogéniques comme les MMPs et les facteurs de croissance chez une variété de cellules dont les lymphocytes, macrophages et cellules endothéliales, favorise la formation de nouveaux vaisseaux afin d'irriguer la plaque athérosclérotique. Effectivement, les MMPs incluant MMP-1, MMP2, MMP3 et MMP9 dégradent la matrice extracellulaire afin que les cellules endothéliales activées prolifèrent au sein de la matrice riche en facteurs de croissance dont VEGF, FGF et PAF (229, 230, 350, 351). Ainsi, ces événements amènent à la formation d'une plaque fibrolipidique composée d'une

panoplie de cellules comme les cellules spumeuses, CML, leucocytes ainsi que des particules riches en lipides et des protéines de la matrice extracellulaire (213).

2.4.3.2 L'instabilité de la plaque et l'athéro-thrombose

La rupture de la plaque est l'évènement causal principal responsable de l'athéro-thrombose. En effet, la rupture de la plaque entraine l'exposition de facteurs thrombogéniques dont l'exposition des protéines de la matrice extracellulaire, surtout le collagène, permettant l'adhésion des plaquettes recrutées à la lésion vasculaire et la formation éventuelle du thrombus (352). L'interaction du CD40L avec le CD40 des cellules leucocytaires, cellules endothéliales et CML, favorise l'instabilité de la plaque en induisant la sécrétion accrue des MMPs (MMP1, MMP2, MMP3, MMP8, MMP9 et MMP13). En effet, les MMPs dégradent la matrice extracellulaire dont le collagène interstitiel menant à la fragilisation de la chape fibreuse et la rupture de la plaque. Par conséquent, les composantes de la matrice sous-endothéliale exposées comme le collagène extracellulaire incitent l'adhésion des plaquettes au site de rupture, menant alors à la formation du thrombus (234, 236, 344, 353-355). L'interaction CD40L/CD40 provoque aussi la génération du TF au niveau de la lésion athérosclérotique. En effet, la signalisation du CD40 de différentes cellules vasculaires dont les macrophages, cellules endothéliales et CML induit une augmentation de l'expression du TF (231, 237, 356). Le niveau élevé de TF favorise le potentiel thrombogénique du site de rupture par l'activation de la voie extrinsèque de la cascade de coagulation ainsi que l'activation plaquettaire suite à la contribution du TF à la formation de la thrombine, un puissant activateur plaquettaire et un facteur pro-coagulant. D'ailleurs, la liaison du CD40L aux cellules endothéliales diminue l'expression de la thombomoduline, une molécule inactivatrice de la thrombine (232, 357).

La liaison du CD40L sur les plaquettes joue un rôle crucial dans la formation du thrombus dans l'athérothombose. Ainsi, l'interaction du CD40L avec le CD40 des plaquettes induit l'activation, la sécrétion et le changement de forme plaquettaire (278, 296, 297). Notre laboratoire a démontré que l'interaction du CD40L avec le CD40 des plaquettes potentialise l'agrégation plaquettaire en présence de doses sous-optimales d'agonistes plaquettaire *via* l'activation de deux voies indépendantes, p38 MAPK et NF-κB. Cela peut favoriser la formation de thrombus (294, 295). D'ailleurs, le sCD40L augmente l'activation plaquettaire et la formation de thrombus chez les souris WT, mais pas chez les souris $CD40^{-/-}$ (294). De plus,

l'interaction du CD40L avec l'$\alpha_{IIb}\beta_3$ des plaquettes permet la stabilisation du thrombus (191, 242). Or, l'interaction du CD40L avec $\alpha_5\beta_1$ pourrait participer à l'activation plaquettaire (249). Enfin, les plaquettes sont majoritairement à l'origine de la présence du sCD40L en circulation (95% du sCD40L proviennent des plaquettes). Le sCD40L constitue un prédicteur de plusieurs pathologies cardiovasculaires considérant la contribution importante du CD40L dans l'athéro-thrombose. Effectivement, plusieurs études cliniques associent le taux de sCD40L circulant à l'occurrence des maladies cardiovasculaires dont l'ACS, IM et l'angine. D'ailleurs, des patients souffrant de maladies cardiovasculaires présentent un taux élevé de sCD40L circulant, comparativement à des sujets sains (358-364). Le taux élevé de sCD40L est aussi associé à la resténose chez les patients ayant subi une angioplastie (365, 366).

Chapitre 3 : Le NF-κB plaquettaire

3. Revue de littérature

Ce chapitre sera couvert par une revue de littérature publiée en 2019 dans le journal *International Journal of Molecular Sciences*.

Role of NF-κB in Platelet Function

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Abstract: Platelets are megakaryocyte-derived fragments lacking nuclei and prepped to maintain primary hemostasis by initiating blood clots on injured vascular endothelia. Pathologically, platelets undergo the same physiological processes of activation, secretion, and aggregation yet with such pronouncedness that they orchestrate and make headway the progression of atherothrombotic diseases not only through clot formation but also via forcing a pro-inflammatory state. Indeed, nuclear factor-κB (NF-κB) is largely implicated in atherosclerosis and its pathological complication in atherothrombotic diseases due to its transcriptional role in maintaining pro-survival and pro-inflammatory states in vascular and blood cells. On the other hand, we know little on the functions of platelet NF-κB, which seems to function in other non-genomic ways to modulate atherothrombosis. Therein, this review will resemble a rich portfolio for NF-κB in platelets, specifically showing its implications at the levels of platelet survival and function. We will also share the knowledge thus far on the effects of active ingredients on NF-κB in general, as an extrapolative method to highlight the potential therapeutic targeting of NF-κB in coronary diseases. Finally, we will unzip a new horizon on a possible extra-platelet role of platelet NF-κB, which will better expand our knowledge on the etiology and pathophysiology of atherothrombosis.

Keywords: NF-κB; platelet; signaling pathways; inflammation; thrombosis

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1. Introduction

Cardiovascular disease is the largest cause of death globally. According to the report from the World Health Organization (WHO), the death rate caused by cardiovascular diseases worldwide is estimated at 17.3 million people in 2008, accounting for 30% of deaths globally. In addition, it is estimated that this number may increase to 23.6 million people [1]. Cardiovascular disease is often caused by thrombotic events such as coronary heart disease. Platelets are major players in the occurrence of cardiovascular diseases since they are involved in various thrombo-inflammatory diseases, particularly atherosclerosis and its progression to atherothrombosis in acute coronary syndrome (ACS) patients [2–5]. Indeed, platelets mediate primary hemostasis and formation of thrombosis, however, thrombosis can become pathological when it occurs, mainly, after the rupture of an atherosclerotic plaque. Thus, following an atherosclerotic vascular lesion, exposed sub-endothelial collagen, alongside thrombin and adenosine diphosphate (ADP) production, triggers platelet activation by interacting with several receptors expressed on platelets such GPIb-IX-V, GPVI-FcRγ, protease activated receptors (PARs), P2Ys, and integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ et $\alpha_{IIb}\beta_3$). Consequently, the "inside-out" signaling of the $\alpha_{IIb}\beta_3$ integrin, in response to platelet activation, leads to platelet aggregation thanks to the binding of $\alpha_{IIb}\beta_3$ to fibrinogen promoting the formation of a thrombus [6,7]. Such a process could induce a partial or complete occlusion of the blood vessel, which leads to a decrease or blockage of the blood flow and thus becomes a cause of occurrence of ischemia or infarction of an irrigated organ such as the heart. Furthermore, the release of a plethora of inflammatory mediators by activated platelets such as soluble P-selectin, soluble CD40 ligand (sCD40L, also known as CD154) and interleukin-1 beta (IL-1β), which interact with cells that mediate inflammation such as circulating leukocytes, endothelial cells, and progenitor cells, and thereby aggravate inflammatory responses [8,9]. At the molecular level, several pathways promoting the initiation and progression of inflammatory diseases [10] are regulated by NF-κB, a prominent transcription factor extensively described in innate and adaptive immune cells as a governor of essential physiological processes including cell survival, proliferation, and activation. In terms of atherosclerosis, vascular fatty plaques host a pro-inflammatory milieu partly kindled by platelets in which NF-κB appears as an important regulator of inflammatory and thrombotic responses, albeit to a less clear extent in terms of mechanics and functions, compared to NF-κB expressed in various cells [11]. Indeed, the activation of NF-κB in

endothelial cells in response to an inflammatory environment triggers the expression of adhesion molecules, which increases binding and transmigration of leukocytes and platelets, while unleashing their thrombogenic potential. The activation of NF-κB in monocytes is required for their differentiation to macrophages and contributes to the expression of tissue factor and release of inflammatory cytokines. In neutrophils, NF-κB activation extends their survival and induce the expulsion of neutrophil extracellular traps (NETs), which exert antibacterial functions and triggers a strong coagulatory response and may induce the formation of microthrombi. NF-κB plays a crucial role in lymphocyte proliferation and cytokines production, which promotes enhanced inflammatory and thrombotic response by recruiting platelets [11]. In this review, since NF-κB is a central hub in thrombo-inflammatory reactions, we aim to enhance our knowledge on the less-characterized role of NF-κB in platelets. Moreover, we will harness this novel non-genomic role of NF-κB at in pinpointing its implications in cardiovascular diseases like atherosclerosis and atherothrombosis in ACS. Furthermore, we will highlight the effects of several active compounds on NF-κB, thereby bridging to its potential therapeutic utility particularly at the level of platelets. Finally, we will widen our compass to include a potential extra-platelet role of platelet NF-κB, which might oblige us to modify our understanding not only of the role of platelets but also of the progression of atherothrombotic diseases.

2. The Genomic Role of NF-κB

NF-κB proteins exist in the cytoplasm as inactive dimers associated with the inhibitor IκB subunits (IκBα and IκBβ) which prevent their activation. Of the two subunits, IκBα is the most represented. In most cells, those dimers are formed by five Rel/NF-κB DNA-binding subunits: p50 NFκB1, p65/REL A, cRel, NFκB2/p52, and Rel B. NF-κB is regulated by the multi-subunit IκB kinase (IKK) [12], which catalyzes the phosphorylation of IκB causing its proteasome-mediated degradation. Among IKK subunits, IKKβ is the most active. Indeed, IKKβ deficiency in embryonic mice fiercely prevents NF-κB activation [13]. Once IκB is degraded, active NF-κB is liberated, after which it translocates to the nucleus to transcribe pro-inflammatory and pro-survival genes in two distinctive pathways, canonical and non-canonical, implicating p50/RelA and p52/RelB, respectively [14] (Figure 1). In such cellular settings, the role of NF-κB is genomic and well-documented since its discovery in immune cells over 30 years ago [15–23].

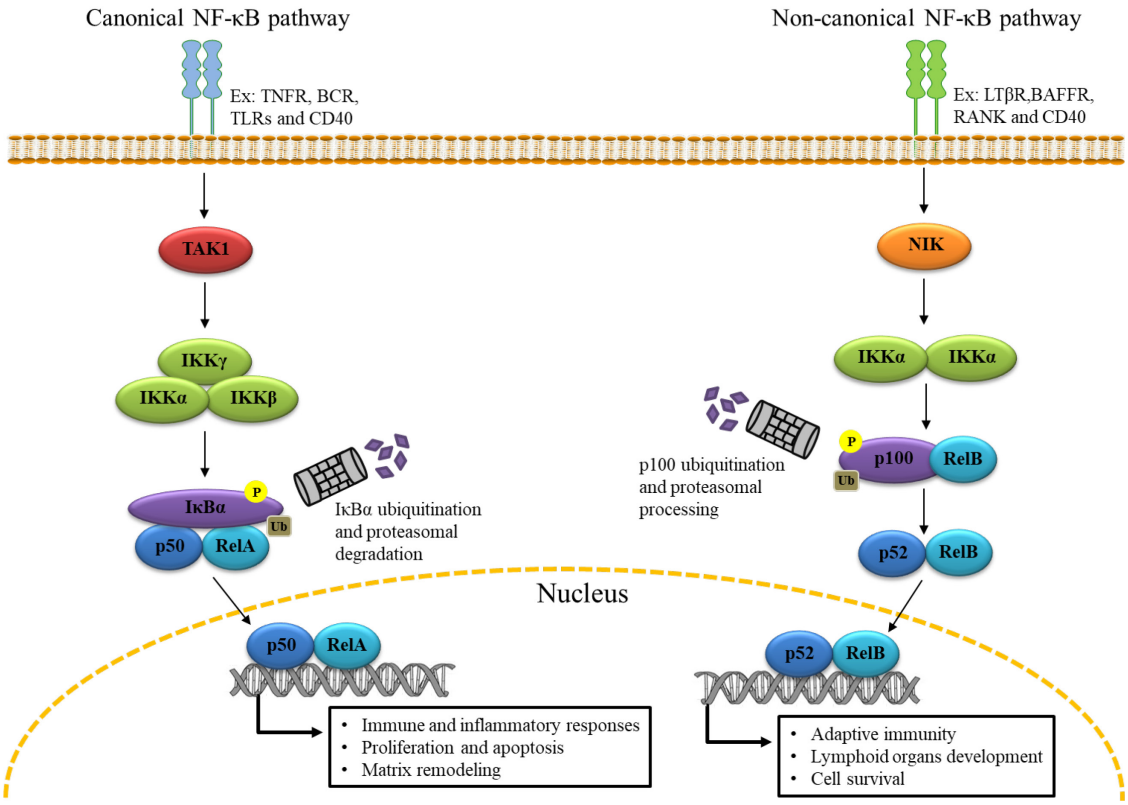

Figure 1. NF-κB Activation: Canonical and non-canonical pathways. The activation of the canonical pathway is triggered by various receptors such as the tumor necrosis factor receptor (TNFR) and Toll-like receptors (TLRs) or the B-cell receptor (BCR). This pathway involves activation of the IκB kinase (IKK) complex (IKKα, IKKβ, and IKKγ) by TAK1 and IKK-mediated IκBα phosphorylation. IκBα phosphorylation induces its ubiquitination and degradation by the proteasome leading to the nuclear translocation of p50/RelA dimers. The activation of the non-canonical pathway is activated by different receptors such as the lymphotoxin-β receptor (LTβR) and the B-cell activating factor receptor (BAFFR). This pathway relies on the activation of NF-κB-inducing kinase (NIK) an IKKα, which leads to the phosphorylation and ubiquitination of p100 and subsequently the processing of p100 by the proteasome to generate transcriptionally active p52/RelB dimers.

3. NF-κB Expression in Platelets

Acellular fragments are derived from megakaryocytes, platelets are devoid of nuclei, yet they express IKK, IκB, and NF-κB [24]. Likewise, NF-κB is expressed in other anucleated cells like mature erythrocytes [25]. Specifically, Liu et al. were the first to demonstrate the expression of NF-κB in platelets in 2002, revealing that thrombin-induced platelet activation triggers the

degradation of IκBα following its serine 32 residue phosphorylation [26]. Afterwards, several other studies corroborated the same finding [24,27–29], suggesting that platelets have a much more complex nature than simply being the "remnants of megakaryocytes". This is supported by the identification of several other transcription factors in platelets such as peroxisome proliferator–activated receptors (PPARs) [30–33], retinoid X receptor (RXR) [34,35], glucocorticoid receptor (GR) [36,37], STAT3 [38], as well as spliceosomes, transcriptomes, messenger RNAs (mRNAs), microRNAs (miRNAs) [39–42], and other diverse components, which partake in translational execution in a signal-driven manner [43]. The exact role of platelet NF-κB, however, remains elusive.

4. NF-κB Functions in Platelets

As is the case in other cells, NF-κB signaling in platelets involves IKKβ phosphorylation, IκBα degradation, and p65 phosphorylation [24,44,45]. However, unlike other cells, the culminating events of NF-κB signaling in platelets remain partially understood. For this purpose, multiple studies were performed utilizing either pharmacological inhibitors of NF-κB such as BAY 11-7082 or knockout mice to unravel the role of NF-κB in platelets, specifically in the context of their survival/apoptosis, priming, activation, and aggregation.

4.1. Platelet Survival and Apoptosis

The least ventured in platelet NF-κB research is its function in survival and apoptosis, namely because platelets are short-lived with a lifespan close to 5 days in mice and 10 days in humans [46]. A report by Dowling et al. reasons out this brief lifespan using a bio-mathematical model, demonstrating that early platelet senescence is an internally controlled mechanism rather than being the result of multiple deteriorating hits [47]. More specifically, it is proposed that early after shedding from megakaryocytes, platelets encompass enough B-cell lymphoma-extra large (Bcl-xL), a protein of the antiapoptotic Bcl-2 family, to overthrow the effects of Bax and Bak, pro-apoptotic molecules, which disrupt mitochondrial membrane permeability and trigger caspase-driven apoptosis. Later in their lifespan, however, and in the absence of external death signals, platelets are unable to synthesize more Bcl-xL, and thus the more labile Bak and Bax regain their status, driving platelets to their final demise-being washed away from the blood stream [48]. Although the proteasome might be a major contributor [49], the players and exact

mechanisms that control this putative internal timer between life and death are still partially understood, especially given that caspase-independent pathways are also recorded in platelets [49,50]. In multiple cell types, NF-κB confers a pro-survival role by promoting G1-to-S phase cell cycle progression [51], inducing the transcription of several above-mentioned cytokines and growth factors, and upregulating anti-apoptotic proteins (XIAP, Bcl-xL, Bcl-2, A1/Bfl-1) [52,53]. In platelets, however, the role of NF-κB in the same context is still elusive. A recent study showed that upon treatment of platelets with NF-κB inhibitors, a significant increase of intracellular calcium was recorded in parallel with (i) decreased sarco/endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA) activity, (ii) increased ER stress, (iii) pronounced mitochondrial membrane depolarization and mitochondrial permeability transition pore (MPTP) formation, (iv) downregulated Bcl-2 levels, and (v) increased caspase activity and apoptosis. Upon pharmacologically preventing ER stress, however, MPTP formation and apoptosis were reversed. This string of events suggests that NF-κB might moderate calcium hemostasis in platelets, possibly by regulating the function of ER membrane-implemented SERCA, thereby favoring survival and preventing ER stress-triggered mitochondrial-driven platelet apoptosis [54]. Aside from the apoptosis pathway, inhibiting GPIbα shedding in platelets was able to reduce platelet clearance [55–57]. Indeed, the shedding of GPIbα is a physiological mechanism mediated by a disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) that takes place constantly on the platelet surface [58]. However, GPIbα shedding is linked to an increase in platelet clearance. Indeed, under physiological shear stress, the binding of Von Willebrand Factor (VWF) with GPIbα induces unfolding of the mechanosensory domain (MSD) on the platelet surface, thereby boosting shedding of GPIbα and triggering GPIb-IX signaling, leading to rapid platelet clearance [59,60]. NF-κB could contribute to GPIbα shedding in platelets; for instance, in response to thrombin stimulation, IKKβ-deficient platelets were unable to shed GPIbα. However, in response to ADP or collagen stimulation, GPIbα shedding was unaffected; this might suggest that IKKβ is uniquely implicated in thrombin-induced GPIbα shedding [61].

4.2. Platelet Activation and Priming

Following their stimulation with specific agonists such as thrombin or collagen, platelets undergo activation in a series of processes including shape change, cytoskeleton rearrangement,

and organelle centralization. In parallel, the release of dense granules content including ADP occurs, triggering further platelet recruitment and activation. More specifically, ADP activates platelets by binding its receptors, $P2Y_1$ and $P2Y_{12}$, and inducing the expression of platelet P-selectin. Similarly, the release of thromboxane A2 (TXA_2) enhances platelet activation and recruitment and leads to platelet aggregation [62,63].

NF-κB inhibitors prevent platelet activation following stimulation with several agonists, suggesting that NF-κB in platelets functions rather non-genomically inducing platelet activity.

4.2.1. Thrombin-Activated Platelets

Thrombin is a key activator of human platelets by binding PAR-1 and PAR-4 receptors, promoting the expression of several inflammatory mediators such as P-selectin, IL-1β, and CD40L [64]. Moreover, thrombin triggers ADP release, TXA_2 production, and IκBα phosphorylation [26]. Thrombin-stimulated platelets pretreated with BAY 11-7082, an irreversible inhibitor of IKKβ phosphorylation, or Ro 106-9920, a selective inhibitor of IκBα ubiquitination, exhibit drastically low expression of P-selectin, TXA_2 production, and ADP release [27]. Interestingly, the phosphorylation of ERK, a mediator of granule secretion, is inhibited, too [65]. Karim et al. further explain IKKβ implication in platelet secretion through its ability to phosphorylate synaptosome-associated protein-23 (SNAP-23), a member of membrane proteins complex called soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs), which regulates granule secretion. Indeed, SNAREs play a major role in the fusion of platelet granules with the platelet membrane. In fact, when platelets are activated in response to thrombin stimulation, vSNAREs (vesicular SNAREs) such as VAMP-8 (vesicle-associated membrane protein–8) that present on platelet granules surface bind to tSNAREs (target SNAREs) such as Syntaxin 11 expressed on the platelet membrane. The binding is mediated mainly by SNAP-23. Thus, SNAP-23 phosphorylation in thrombin-stimulated platelets facilitates the fusion between granules and plasma membrane for cargo release [66]. In contrast, the use of selective inhibitors of IKKβ (BMS-345541 [67], TPCA-1 [68], and BAY-11-7082) prevents thrombin-stimulated SNAP-23 phosphorylation in a dose-dependent manner [66]. Additionally, the same group shows that thrombin-stimulated platelets from IKKβ knockouts have a lower capacity to release alpha, dense, and lysosomal granules content by an overall decrease of 30%. Wei et al. [61], who demonstrated that platelet IKKβ

deficiency exhibits decreased secretion and activation confirmed this observation. Interestingly, a recent study showed that the inhibition of MALT1, an upstream regulator of IKK complex and a member of the functional proteasome CARMA/MALT1/Bcl10 (CBM) complex, prevented platelet activation and secretion by abrogating SNARE formation [69]. Such findings suggest that NF-κB could contribute to platelet degranulation in platelet secretion.

It is suggested that PAR-4, a thrombin receptor, is the main activator of NF-κB in platelets through a mechanism involving sphingomyelinase (nSMase), an enzyme which catalyzes the transformation of sphingomyelin into ceramide and phosphorylcholine and which notably partakes in macrophage NF-κB activation [70]. More specifically, the binding of thrombin to platelet PAR4 induces nSMase activation, which increases C24:0-ceramide levels. This is followed by the activation of p38 MAPK, which in turn initiates NF-κB and platelet activation [71].

4.2.2. Collagen-Activated Platelets

In addition to thrombin, collagen is another platelet agonist discovered to phosphorylate IκBα and thus activate NF-κB [24]. Collagen interacts with platelet glycoprotein receptors, GP1b and GPVI, triggering intracellular signaling, promoting integrin αIIbβ3 receptor activation, and inducing the release of secondary mediators like ADP and TXA_2. The use of BAY-11-7082 prior to platelet stimulation with collagen elevates cyclic AMP and enhances vasodilator-stimulated phosphoprotein (VASP) phosphorylation, therefore, suppressing TXA_2 formation, ATP release, P-selectin expression, and intracellular Ca^{2+} immobilization [29]. Of note, VASP, an actin-and prolifin-binding protein and a substrate of cAMP-dependent protein kinase A (PKA) [72] is a major negative regulator of platelet secretion and adhesion [73]. Oppositely, a study by Gambaryan et al. suggested that upon IκBα degradation, PKA dissociates from NF-κB and phosphorylates VASP. In addition, following platelet pre-treatment with an IKK inhibitor VII, a competitive reversible inhibitor of IKKβ, VASP phosphorylation, and its platelet-inhibitory effect is lost after stimulation with both, collagen and thrombin [28]. However, this study might be demonstrating a peculiar negative feedback signaling to avert excessive platelet activation. Nevertheless, the results require further explanation.

4.2.3. CD40L-Primed Platelets

As mentioned before, platelet agonists trigger the expression of several inflammatory mediators, among which is CD40L, a member of the TNF family mainly expressed in T lymphocytes and activated platelets. CD40L rapidly appears on the platelet surface following activation, upon which it is subsequently cleaved generating a soluble fragment of 18-kDa, termed sCD40L [74]. Activated platelets constitute the primary source of sCD40L, accounting for >95% of its plasmatic concentration [75,76]. Elevated levels of sCD40L are now considered reliable predictors of cardiovascular diseases [76–85]. The discovery of new CD40L receptors ($\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_M\beta_2$) [86–88], in addition to its classical/high-affinity receptor CD40[89], adds complexity to the diverse interplays to which CD40L takes part in cells in general and platelets in particular.

The receptor CD40, constitutively expressed on platelets, lacks intrinsic signaling activity and needs to recruit adaptor molecules, such as the (TNFR)-associated factors (TRAFs) that bind to the cytoplasmic domain of CD40 and subsequently recruit kinases and other effector proteins responsible for transducing signals [90]. CD40 can bind five of the seven TRAF family members (TRAF1, 2, 3, 5, and 6). The cytoplasmic domain of CD40 has a proximal TRAF6 binding site and a more distal TRAF2/3/5 binding site. TRAF1 only binds to CD40 when CD40 signaling is already active and acts as a regulator rather than an activator of CD40 signaling [91]. The binding of TRAF5 to CD40 is still debated, and its role in CD40-mediated signaling remains controversial [92]. Overall, CD40-TRAF signals stimulate kinase activation and gene expression and induce the production of antibodies and a variety of cytokines, expression and upregulation of adhesion molecules, and protection or promotion of apoptosis. These various pathways can culminate in either the induction or inhibition of biological functions.

Conversely, platelets express the receptors $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ in their inactive form, whereupon platelet activation by ligands such as fibrinogen and fibronectin, undergo certain conformational changes including the interaction through their cytoplasmic tails with intracellular signal transduction proteins such as as FAK, Src, and talin rendering them active [93]. CD40L can bind both inactive and active forms of $\alpha_{IIb}\beta_3$, yet can only bind the inactive form of $\alpha_5\beta_1$ [94].

In platelet physiology, our laboratory showed that CD40L alone induced IκBα phosphorylation and NF-κB activation exclusively through platelet CD40 receptor [95]. We also showed that sCD40L, in the presence of sub-optimal doses of platelet agonists like collagen and thrombin, significantly increased platelet activation and aggregation through an NF-κB-

independent CD40/TRAF-2/Rac-1/p38 MAPK axis [9]. The first result explains the priming effects of CD40L, priming being a pre-activation process that ultimately prepares platelets for aggregation through a series of molecular events including P-selectin expression and lamellepodia formation, all of which we found concurrent with NF-κB activation. The second result, however, might explain that NF-κB activity driven by sCD40L priming capacity bridges to CD40/TRAF-2/Rac-1/p38 MAPK axis-induced platelet aggregation. This was evident in first, following the use of IKK inhibitor VII, platelet priming, and potentiation of aggregation were significantly diminished, and second, IκBα and p38 MAPK phosphorylation were found to be independent upon platelet pre-treatment with either of their inhibitors followed by sCD40L stimulation. Furthermore, our recent study dug deeper into the unique NF-κB signaling pathway, showcasing that CD40L activated NF-κB through CD40 and TAK-1 and endorsing that TAK-1 is upstream of NF-κB in CD40L signaling as its inhibition completely eliminated NF-κB activation [95]. Nevertheless, a study by Kuijpers et al. [96] contradicted our results, demonstrating that CD40L enhanced collagen-induced platelet-platelet interactions by supporting integrin αIIbβ3 activation, platelet secretion, and thrombus growth via PI3Kβ but not CD40 and IKKα/NFκB. Still and all, the paper claimed that first, CD40 deficiency led to increased integrin αIIbβ3 expression, which contradicts the results of a third study by Inwald et al. [97] and second, P-selectin expression and phosphatidylserine exposure were not affected by CD40L preincubation, thereby also contradicting several other studies [9,97–99].

In summary, Figure 2 resumes our previous findings that established the link between sCD40L, enhanced platelet reactivity and thrombosis involving its main receptor CD40 binding to an adaptor protein the (TNFR)-associated factor-2 (TRAF2) and downstream signaling via Rac1/p38-MAPK [9]. We have also revealed that sCD40L is a potent activator of NF-κB [100], which primes platelets through CD40 signaling via the transforming growth factor-B (TGF-B)-activated Kinase (TAK1) [95].

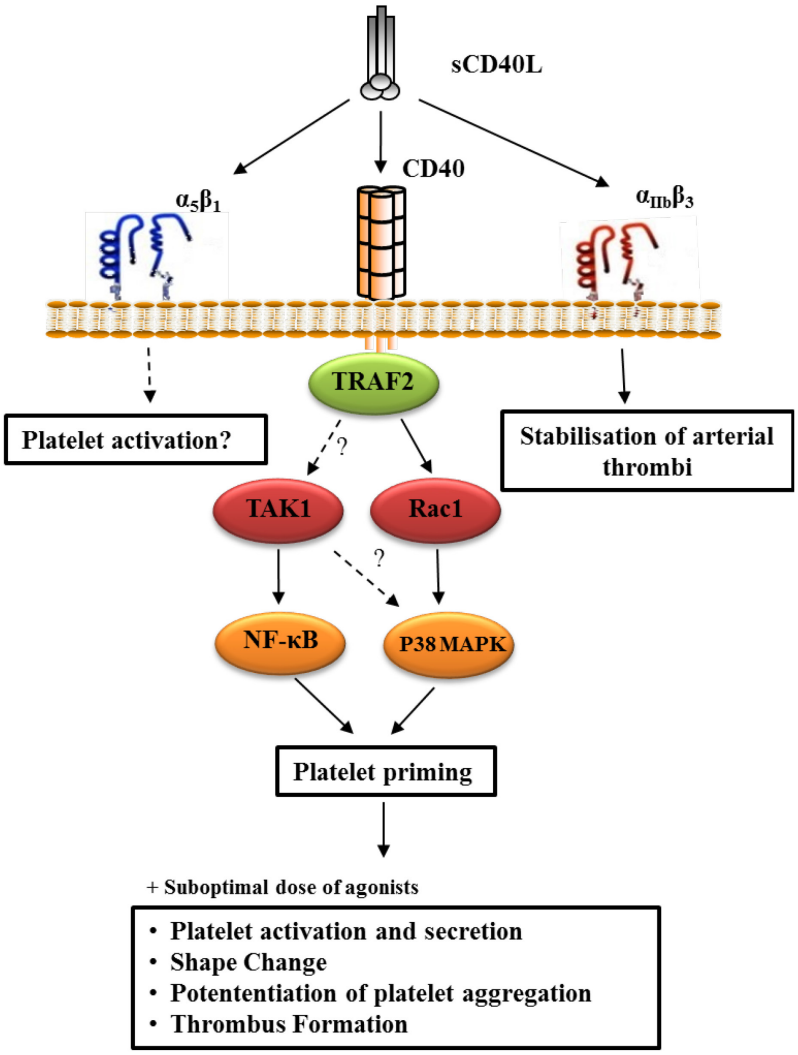

Figure 2. Proposed pathways of sCD40L/CD40 function in platelets**.** sCD40L enhancement of platelet reactivity or priming involves its main receptor CD40 binding to an adaptor protein the (TNFR)-associated factor-2 (TRAF2) and downstream signaling Rac1/p38-MAPK and TAK1/NF-κB. In response to suboptimal doses of agonists, primed platelets potentiate platelets function, which can promote thrombus formation.

4.2.4. TLR Ligand-Activated Platelets

Involved in immune and inflammatory responses, the family of toll-like receptors (TLRs) is expressed on platelets [101,102]. Each TLR identifies different types of pathogen-associated molecular patterns (PAMPs) or ligands present on viruses, bacteria, and fungi. Among TLRs, TLR2, and TLR4 are the most involved in platelet activation, as translated by their increased

expression [103]. Following platelet TLR 1/2 stimulation with Pam3CSK4, a synthetic agonist of TLR2/TLR1, αIIbβ3 activation, and P-selectin expression are increased, leading to hemostatic and inflammatory responses. This coincides with an elevation in PI3K/Akt, ERK1/2, and p38 activity, P2 × 1-mediated Ca^{2+} mobilization, TXA_2 production, and ADP release. As for TL4, platelet activation with lipopolysaccharide (LPS), a TLR4 agonist, triggers platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and cGMP-dependent protein kinase pathways [104]. It is also well documented that TLRs activate NF-κB in nucleated cells and drive the production of proinflammatory cytokines like IL-1β and TNFα [105,106]. A study by Rivadeneyra et al. demonstrated that TLR2 and 4 agonists induced platelet activation responses through NF-κB. In parallel, IκBα degradation and p65 phosphorylation were observed. In a relatable manner, platelet treatment with BAY 11-7082 or Ro 106-9920 impaired TLR-mediated platelet activation [44]. Other studies on chicken thrombocytes, the hematological equivalents to mammalian platelets, highlighted the role of NF-κB in TLR signaling, showing that inhibition of IKK with BMS345541 results in a significant reduction in thrombocytes' secretory profile [107,108].

4.2.5. AGE-Activated Platelets

The binding of advanced glycation end-products (AGEs) to their receptor, RAGE, is believed to play an important role in the pathophysiology of several cardiovascular diseases such as heart failure and coronary disease, as well as peripheral artery diseases observed in diabetes [109]. RAGE is expressed on a plethora of cells such as macrophages, monocytes, endothelial cells, neutrophils, lymphocytes, and platelets [110–113]. In platelets, RAGE activation increases P-selectin expression on the platelet membrane, hence promoting platelet activation [111]. Knowing that RAGE induces NF-κB activation leading to the secretion of pro-inflammatory cytokines in monocytes [114] and that platelets possess all elements of NF-κB signaling cascade downstream RAGE, including ERK and p38 MAP kinase [115], it is possible that RAGE might induce NF-κB activation in platelets, however, further investigations are required to confirm this theory.

4.2.6. Epinephrine-Primed Platelets

Secreted by the adrenal gland, epinephrine is a hormone with alpha- and beta-adrenergic sympathomimetic activities [116,117]. Alone, epinephrine does not induce platelet activation through platelet alpha 2-adrenergic receptors. However, when present with other platelet agonists, it can potentiate their activation and aggregation responses, hence being a platelet "primer" like CD40L mentioned above [118]. This priming action is primarily mediated by IKK activation, which culminates in inducing p38 and PKA signaling as well as activating $\alpha_{IIb}\beta_3$ integrin receptor [11]. In platelet rich plasma pre-treated with BAY 11-7082 and Ro 106-9920, the potentiating action of epinephrine is impaired in the initial stages of aggregation inclusively, further evidencing the involvement of NF-κB in moderating the early stages of platelet activation [27].

4.2.7. ADP-Activated Platelets

ADP is the earliest identified platelet agonist which exerts its effects through three purinergic receptors, two of which are G protein-coupled: (i) $G_{\alpha q}$ coupled-P2Y1, (ii) $G_{\alpha i}$-coupled P2Y12, and Ca^{2+} channel P2 × 1 [119]. Some of the actions mediated by ADP on platelets include adhesion, shape change, granule secretion, Ca^{2+} influx and intracellular mobilization, adenylyl cyclase inhibition, TXA_2 production, and aggregation induction [120,121]. The role of NF-κB in regulating the initial stages of ADP-induced platelet activation is demonstrated upon pre-incubation of platelet rich plasma with BAY 11-7082 and Ro 106-9920 followed by ADP treatment. Resulting is a significant impairment of early platelet aggregation [27].

4.3. Platelet Aggregation

Platelet aggregation is the ultimate process in primary hemostasis, wherein platelets clump together creating a stable hemostatic plug following their endothelial damage. The cross-linking of essentially activated αIIbβ3 integrin receptors on adjacent platelets by soluble fibrinogen mediates this process. In fact, platelets express an inactive form of αIIbβ3 integrin, which upon platelet activation by thrombin, collagen, and ADP, intracellular signals are triggered, leading to conformational changes in αIIbβ3 that transit the receptor from an inactive low affinity state to an active high affinity state. This "inside-out" signaling mechanism allows the full exposure of fibrinogen binding sites in αIIbβ3 integrin.

The use of inhibitors of IKKβ phosphorylation highlighted the influence of NF-κB activation on platelet aggregation. Therein, treating platelets with BAY 11-7082 or Ro 106-9920

decreases fibrinogen binding following thrombin and collagen stimulation, which inhibits αIIbβ3 inside-out signaling, platelet aggregation, and clot retraction [27,29,71]. Andrographolide, an active ingredient found in the leaves of a medicinal herb, is another less-utilized potent NF-κB inhibitor, which prevents p65 phosphorylation in collagen-stimulated platelets NF-κB and interferes with their function. Although andrographolide is shown to inhibit platelet aggregation via eNOS activation and inhibition of both, PLCγ2–PKC and PI3kinase/Akt-MAPKs pathways, it is unknown yet if this is the result of its NF-κB inhibitory functions [45,122]. As aforementioned, NF-κB signaling has a significant role in platelet activation. Therefore, it is not surprising that the cytoskeletal rearrangements leading to the conformational changes of αIIbβ3 integrin, which grants platelets their activity, do not occur since IKKβ phosphorylation inhibitors dampen platelet activation. Our laboratory showed that the use of IKK inhibitor VII and BAY 11-7082 prevented aggregation of platelets pretreated with sCD40L and stimulated thereafter with a priming dose of collagen [100]. Surprisingly, the study by Gambaryan et al. demonstrated the opposite role of IKKβ inhibitors, which they potentiated platelet aggregation. Nevertheless, the maximum amplitude of aggregation was similar between IKK inhibitor VII-treated platelets and controls following their stimulation with optimal doses of thrombin and collagen. Therefore, a more plausible result would necessitate the stimulation of IKK inhibitor VII-pretreated platelets with a priming/sub-optimal dose of those agonists, in order to better observe the potentiating effect of the inhibitor. In this context, our recent study demonstrated that the aggregation of platelets pretreated with BAY 11-7082 or TAK-1 inhibitors, 5Z-7-Oxozeaenol and Takinib, primed with sCD40L, and then stimulated with sub-optimal doses of thrombin was abrogated completely [95]. Our results also pinpointed the emerging importance of TAK-1 in NF-κB activation in platelets as another attractive therapeutic target in thrombotic diseases.

Similarly, an in vivo study by Karim et al. reported that the inhibition of IKKβ in mice slowed down thrombus formation and increased bleeding time. Moreover, IKKβ knockout mice also showed increased bleeding times, consistent with the effects of pharmacological inhibition [66]. Of note, Wei et al. demonstrated that IKKβ deficiency promoted leukocyte-platelet interaction by delaying ADAM17-mediated GPIbα shedding, therefore, enhancing platelet aggregation [61].

The pro-coagulant activity of NF-κB was also evident in thrombo-inflammatory conditions like sepsis, in which tissue factor was upregulated in parallel with a downregulation of anticoagulation molecules like anti-thrombin and tissue factor pathway inhibitor. Eventually, the pronounced production of thrombi in this condition induces platelet depletion and subsequent bleeding vulnerabilities, which reflects an extreme role of NF-κB in hematological diseases [123,124].

In the context of diabetes, a recent study on diabetic rats and humans demonstrated that the enhanced expression of platelet $P2Y_{12}$, a pro-thrombotic ADP receptor, correlates with increased IκBα phosphorylation and degradation in platelets and megakaryocytes as well as increased p65 expression and binding to $P2Y_{12}$ promoter in megakaryocytes. The study hence suggested that hyperglycemic conditions render NF-κB the mediator of the increased expression of $P2Y_{12}$ in platelets of patients with type 2 diabetes mellitus [125], evidencing further the pro-thrombotic role of platelet NF-κB in platelets.

Alongside NF-κB, other transcription factors regulate platelet aggregation. For instance, PPARγ agonists decrease CD40L and TXA_2 release as well as platelet aggregation by modulating early GPVI receptor signaling and thus inhibiting collagen-mediated activation [126]. Moreover, RXR ligands inhibit TXA_2 and ADP production and platelet aggregation by binding the GTP-binding protein Gq thus preventing its subsequent activation of Rac and aggregation-driving signaling pathways [34].

5. Natural/Pharmacological Compounds and NF-κB

Several studies on pharmacological and natural active compounds, even those specifically targeting platelets, recount off-target effects on cellular rather than platelet NF-κB.

Firstly, aspirin or acetylsalicylic acid (ASA) is a non-steroidal anti-inflammatory and anti-platelet drug. Requiring the lowest dose and used in the prevention of thrombosis, the anti-platelet indication is established through inhibiting the action of cyclooxygenase-1 (COX-1) responsible for TXA_2 secretion [127]. Because of its dual function, ASA is the most studied medicament in terms of its effects on NF-κB. Kopp and Ghosh were the first to demonstrate that sodium salicylate, an ASA derivative, targets the NF-κB pathway by inhibiting IκB degradation and subsequent NF-κB nuclear translocation in activated T cell lines [128]. Later, it was shown in vitro as well that ASA and salicylate mediated the latter effects through IKKβ inhibition

[129]. In vivo, in a rat model of acute pulmonary embolism, ASA reduced the expression of NF-κB in lung tissues dose-dependently [130]. In peripheral blood mononucleated cells (PBMC) of diabetic patients, the daily dosing of ASA significantly decreased NF-κB binding activity to DNA [131]. However, in atherosclerotic patients, NF-κB expression in carotid artery plaques, precisely that of foam and endothelial cells origins, was unaffected by ASA treatment [132], suggesting that ASA effects on NF-κB are cell- and/or pathophysiology-dependent.

Platelet $P2Y_{12}$ receptor antagonists such as ticagrelor and clopidogrel are another family of anti-platelet agents indicated for patients with acute coronary syndrome at risk of ischemic events [133]. In rats with gastric ulcer, ticagrelor impairs NF-κB p65 phosphorylation [134]. In porcine models undergoing coronary interventions, the long-term administration of clopidogrel shows significant reductions in NF-κB activity [135].

Another anti-aggregation agent, cilostazol is a phosphodiesterase (PDE) 3 inhibitor indicated for treating intermittent claudication in the lower periphery. By increasing cyclic adenosine monophosphate (cAMP) levels, it exhibits vasodilator as well as anti-platelet effects [136]. Cilostazol interferes with the transcriptional activity of NF-κB in macrophages after their treatment with TLR ligands, therefore, reducing the generation of TLR-mediated pro-inflammatory cytokines [137]. In platelets, a recent study on hypercholesterolemia rats treated with cilostazol reported enhanced IκBα expression coinciding with NF-κB inhibition, an effect linked to AMP kinase (AMPK) activation. The study went further by attributing to AMPK activation and subsequent NF-κB inhibition a major role in preserving endothelial function by reducing platelet P-selectin and CD40L expression while increasing endothelial nitric oxide synthase activity responsible for anti-platelet nitric oxide production [138]. Another non-selective PDE inhibitor with anti-inflammatory as well as anti-platelet functions, dipyridamole, exhibits inhibitory actions against IKKβ, IκB phosphorylation and degradation, and p65 nuclear translocation in macrophage cell lines [139] and human PBM [140].

Vorapaxar, a PAR-1 antagonist, recently indicated for the prevention of atherothrombotic events in patients with myocardial infarction and peripheral arterial disease [141], also shows off-label NF-κB effects. In endothelial cells stimulated with cholesterol, vorapaxar significantly increased NF-κB expression levels, suggesting that the molecule performs a protective role in atherosclerotic settings [142]. However, and although of a different clinical context pertaining to hypoxic effects on ventricular remodeling, another study showed the exact opposite, as

endothelial cells from hypoxic mice treated with a PAR-1 antagonist demonstrates downregulated NF-κB levels. The same study showed similar results with rivaroxaban, an anticoagulant drug which functions by directly targeting the clotting factor Xa [143]. In another study, rivaroxaban was demonstrated to attenuate deep venous thrombosis in a rat model by targeting NF-κB signaling pathway in endothelial cells, more specifically downregulating IκB levels as well as NF-κB levels and activity and thereby performing anti-inflammatory and pro-fibrinolytic functions [144]. In cardiac fibroblasts stimulated with Angiotensin II to induce structural and functional remodeling mimicking that of heart failure, rivaroxaban diminished NF-κB activity by 82% [145].

Other non-antiplatelet and non-anticoagulant compounds also exhibit modulatory actions against NF-κB. For instance, a recent study demonstrated that nifedipine, a calcium channel blocker, triggers PPARβ/γ activity. Interestingly, PPARβ/γ inhibits NF-κB activation, hence attenuating intracellular Ca^{2+} mobilization, reducing inside-out αIIbβ3 signaling and fibrinogen binding, and preventing platelet aggregation. It is suggested that the inhibitory effect of PPARβ/γ on NF-κB activation following nifedipine treatment is mediated by its regulation of NO/cGMP/PKG1[146]. Other drug activators of PPARs such as statins, fibrates [147], and thiazolidin [148,149] confer anti-aggregation properties seemingly by acting in a similar way and inhibiting NF-κB. A high dose of simvastatin for example significantly diminishes plasma low-density lipoprotein cholesterol (LDL) levels (including oxidized LDL) in parallel to the binding activity of NF-κB in PBMC [150].

Lastly, a few studies on natural molecules offer additional data on NF-κB modulation. Sesamol 3,4-methylenedioxyphenol), a constituent of sesame oil with antiplatelet effects, inhibits NF-κB pathway by inducing cAMP-PKA signaling cascade, which culminates in the inhibition of intracellular Ca^{2+} mobilization and ultimately platelet aggregation [[151]. Vitamin C dose-dependently prevents IκB degradation and NF-κB activation in vitro, thus aiding to reduce inflammation [152]. Vitamin E, another antioxidant, shows similar effects in vitro and in vivo, although it is unclear whether the inhibition is direct or automatically follows a reduction in oxidative stress [153]. Other natural molecules such as β-carotene, *N*-acetylcysteine, selenium, and omega 3 fatty acids have also exhibited indirect inhibition of the NF-κB pathway [11].

Evident above, there is a scarcity of data on drug pharmacodynamics at the level of platelet NF-κB, as compared to NF-κB in other cells. However, this is fathomable, taking into consideration the reasoning of this review article, that is expanding our limited knowledge on the role of NF-κB in platelet functioning. Consequently, one can extrapolate from the studies performed thus far that targeting platelet NF-κB, as demonstrated with cellular NF-κB, might confer a plethora of therapeutic benefits first at the level of platelet survival, priming, activation, and aggregation and second in the context of atherothrombotic coronary artery diseases. Still and all, further research is mandatory to provide us with the facts.

6. The Interplay between mRNA, miRNA, and NF-κB in Platelets

As mentioned in Section 3, platelets harbor both mRNA and miRNA. In fact, RT-PCR and microarray studies of highly purified human platelets corroborate that the platelet proteome is a copy image of its transcriptome, having identified an average of 2500 platelet-expressed mRNA transcripts (approximately 12,500-fold less than nucleated cells) [39,154,155]. Likewise, Landry et al. have shown that human platelets encompass a wide array of miRNAs (miRs) [156], small (about 22 nucleotides) non-coding RNAs derived from long RNA precursors, and Gregory et al. [157] evidenced further a modulatory role of miRNA in controlling platelet activity [156]. Later genome wide profiling of platelet RNA allowed the identification of about 284 platelet-expressed miRNA [158,159]. Other studies provide further insight on the involvement of miRNA in platelet activation by showing a differential up- and down-regulation of 6 miRNAs upon thrombin stimulation [160].

The NF-κB pathway is not only a direct target but also a modulator of several miRNAs. For instance, miR-223, miR-199a, miR-155, and miR-124a inhibit the expression of different IKK subunits, thus preventing NF-κB activity. On the other hand, NF-κB also regulates a few miRNAs such as miR-29b which is implicated in tumor suppression [161]. It is well known that functional RNA, including miRNA, can move intracellularly via exosomal shuttle-like microparticulate vesicles released by the cell [162]. In addition, activated platelets possess the ability to release microvesicles harboring functional miRNA which can be passed to nucleated cells [163] to modulate their pathological states [164,165], which may suggest the presence of an orchestrated interplay between miRNA and NF-κB mediated by platelet microvesicles. This was recently manifested in a murine study on the protective mechanisms of action of thrombin-

activated platelet-derived exosomes in atherosclerosis. In the scope of our topic, however, and considering that platelet NF-κB is found in platelet microparticles [164], one can postulate that in addition to its non-genomic role in platelet survival and activation-aggregation, platelet NF-κB may harbor an extra-platelet genomic role, which unfolds only following platelet microparticles uptake by other nucleated cells.

7. Conclusion

NF-κB has been shown to be implicated in the transcriptional regulation of over a hundred genes, a large number of these genes exhibit pro-inflammatory properties [166]. In nuclei-devoid platelets which play a major role in cardiovascular diseases, NF-κB is shown to have a different non-genomic function, as summarized in Figure 3. Herein, our endeavors in recapitulating diverse data on platelet NF-κB might help to characterize the exceptional function of this protein in platelets. Although emerging studies corroborate that NF-κB has a primordial role in positively regulating platelet survival, priming, activation, and aggregation, further investigations are warranted to fully elucidate the mechanics and roles of NF-κB in those acellular fragments, especially that another perplexing and equally interesting extra-platelet function for platelet NF-κB has recently been fading in and since few studies showcase an opposite role for NF-κB in platelet function and may, therefore, act as a double-edged sword [28,61]. This, and given the fact that the targeting of NF-κB by several active compounds is elucidated to ameliorate diverse pathophysiological conditions, more pre-clinical research might also bestow upon platelet NF-κB a therapeutic potential in cardiovascular diseases. Thus, inhibiting platelet NF-κB may have a high therapeutic potential to treat thrombotic disorders. Because platelet activation is linked to hemostasis, and also has a key role in inflammation and thrombosis, our present review demonstrates that inhibition of NF-κB interferes with platelet function by reducing its thrombogenic potential and holds great promise when compounds that block NF-κB activation are considered for treating various thrombo-inflammatory diseases.

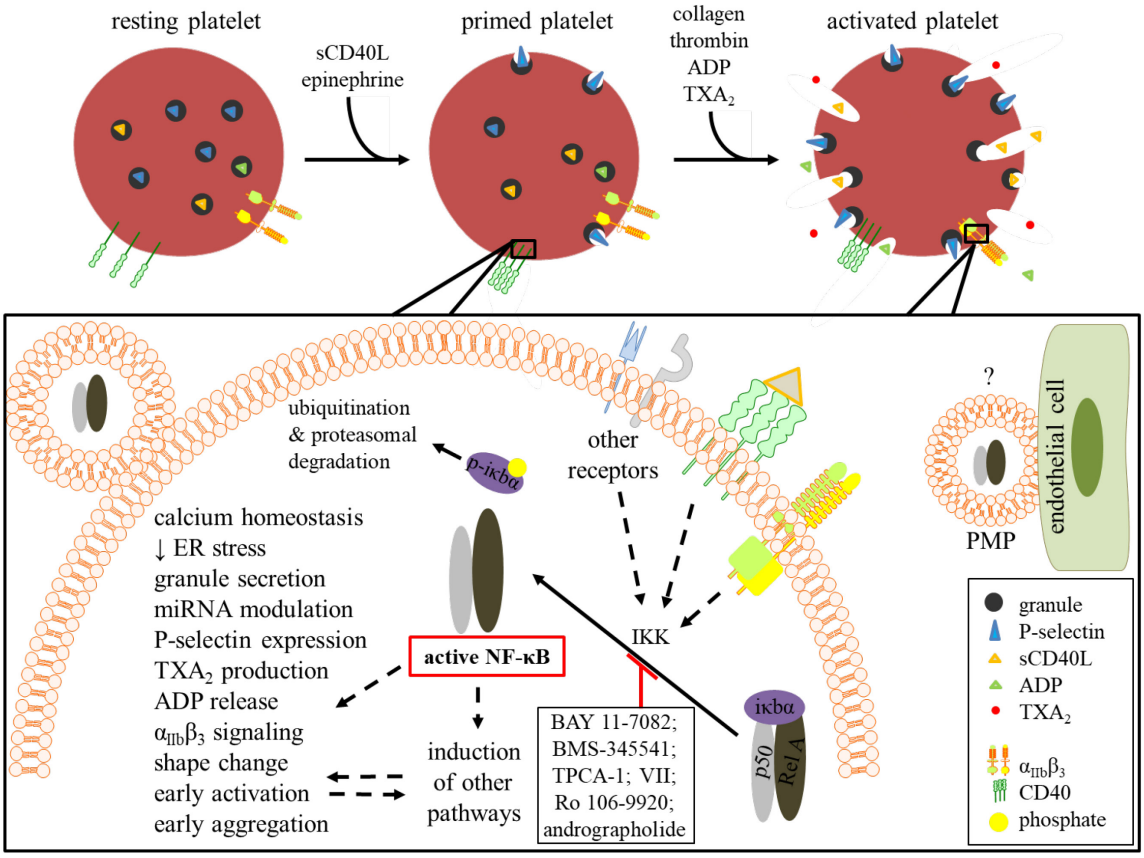

Figure 3. Schematic diagram portraying the role of NF-κB in platelets. Upon ligation of different receptors by priming (sCD40L and epinephrine) and/or activating ligands (collagen; thrombin; ADP; and TxA_2), IKK activation triggers NF-κB pathway. Unlike nucleated cells-originating NF-κB, which translocates into the nucleus and binds genomic DNA, platelet NF-κB confers functions of other nature as shown by the utility of several pharmacological inhibitors of IKK such as BAY 11-7082 and BMS-345541. Activated NF-κB plays a role in platelet survival and platelet priming. Platelet NF-κB might also be involved in regulating miRNA, however, this requires further validation. Although it has been shown that platelet NF-κB carried by platelet microparticles (PMP) is endocytosed by other cells such as endothelial cells, its exact extra-platelet functions are still elusive.

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Chapitre 4 : Les antiplaquettaires

4.1 Les types d'antiplaquettaires

Les plaquettes jouent un rôle fondamental dans l'athéro-thrombose, un phénomène au centre de l'occurrence des coronaropathies, la cause d'environ un tiers des décès dans le monde. De plus, les patients survivant à un événement coronarien présentent un risque de mortalité élevé de 1,5 à 15 fois comparativement aux personnes saines (367). Puisque la composante thrombotique est d'une importance primordiale dans la pathogenèse des événements coronariens, les antiplaquettaires constituent la pierre angulaire des thérapies pour les patients souffrant de coronaropathies ainsi que les patients subissant des procédures de revascularisation comme les interventions coronariennes percutanées (ICP) (368, 369). Les antiplaquettaires diminuent d'environ 25% le risque d'événements ischémiques aigus chez les patients coronariens (370). Quatre types d'antiplaquettaires sont actuellement au cœur de la prise en charge des coronaropathies: les inhibiteurs de COX-1 (aspirine), les inhibiteurs du récepteur de $P2Y_{12}$ (clopidogrel, prasugrel, ticagrelor et cangrelor), les inhibiteurs de PAR1 (vorapaxar) et les inhibiteurs de l'intégrine $\alpha_{IIb}\beta_3$ (abciximab, eptifibatide et tirofiban) (371, 372) (Figure 4.1).

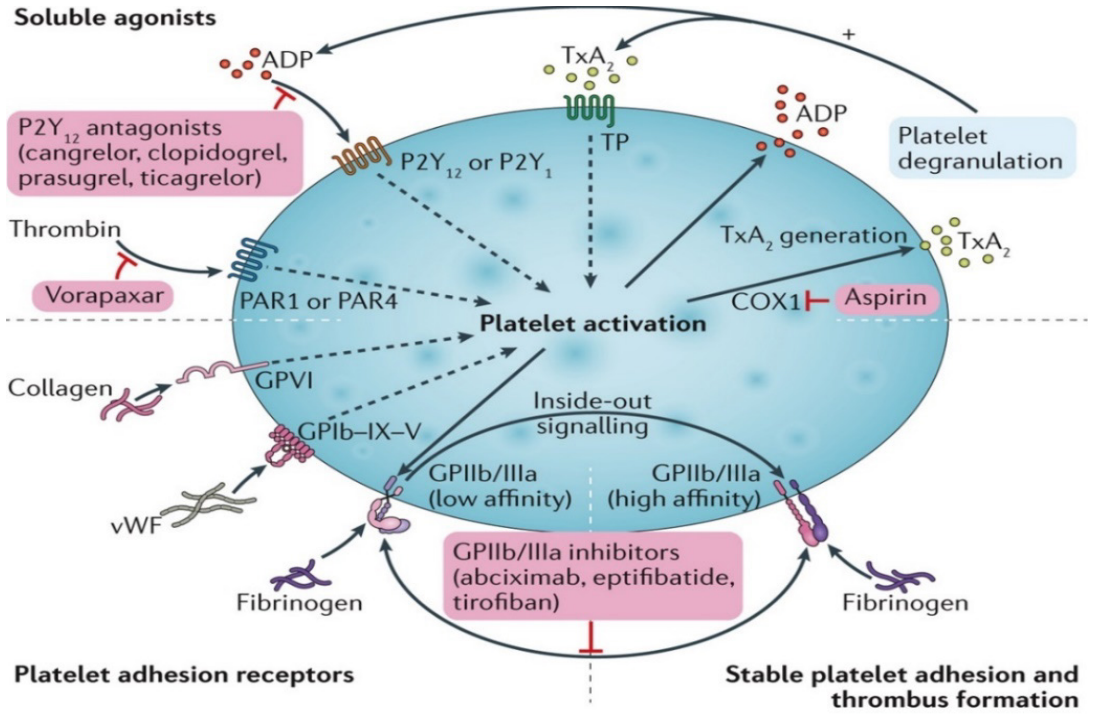

Figure 4.1: Les cibles des antiplaquettaires. McFadyen, JD et al. *Nat Rev Cardiol*. 2018 Mar;15(3):181-191

4.2 L'inhibiteur de COX, l'aspirine ou ASA

Pendant environ trois millénaires, l'écorce de saule était utilisée chez les anciens Égyptiens et Grecques pour réduire l'inflammation et la fièvre. En 1853, Charles-Frédiric Gerharld est le premier à synthétiser l'acide acétylsalicylique (ASA) en sa forme impure et instable. Cependant en 1897, Felix Hoffman, un chimiste allemand œuvrant pour les laboratoires Bayer, synthétise la forme pure et stable de l'ASA et en 1899, Bayer nomme la nouvelle drogue 'aspirine', un nom composé du mot acétyle et *Spiraea ulmaria*, l'arbre qui a permis l'extraction de l'acide salicylique (373). Aujourd'hui, l'ASA ou l'aspirine est le médicament antiplaquettaire le plus répandu pour la prévention et le traitement des thromboses artérielles cardio-cérébrales. L'ASA est capable de réduire efficacement l'incidence des IM non fatal dans la prévention primaire des maladies cardiovasculaires (0.18% ASA vs 0.23% contrôle; $P<0.0001$ par année). Il est aussi efficace dans la prévention secondaire des événements coronariens (4.3% ASA vs 5.3% contrôle par année; $P<0.0001$) et diminue l'incidence des AVC (2.08% ASA vs 2.54% contrôle; $P<0.002$) (374).

4.2.1 Pharmacologie

En 1971, John Vane décrit pour la première fois le mécanisme anti-thrombotique de l'ASA qui est attribuable à l'inhibition de la prostaglandine synthase ou la COX (375). Cette découverte a permis l'émergence des anti-inflammatoires non stéroïdiens (AINS) et autres inhibiteurs de COX. D'ailleurs, en 1982, un prix Nobel lui a été remis pour sa découverte (376). Ainsi, l'ASA inhibe la COX par l'acétylation irréversible d'un résidu sérine, soit la Ser^{529} de la COX-1 et Ser^{516} de la COX-2 (377, 378). En effet, il y a deux isomères de la COX, soit la COX-1 et la COX-2. La COX-1 est constitutivement exprimée sur la plupart des tissus et joue un «rôle domestique» (*houskeeping funtions)*. Elle est impliquée dans la cytoprotection de la muqueuse gastrique, la régulation de la fonction plaquettaire et autres activités physiologiques dont le maintien de la perfusion rénale. La COX-2 a généralement une expression inductible et elle est moins présente au niveau des tissus. Elle s'active durant la réaction inflammatoire afin de catalyser l'AA en une variété de prostaglandine dont le PGE_2 et PGI_2, qui jouent un rôle important dans l'inflammation, la douleur et la fièvre (379, 380). Les plaquettes expriment COX-1 qui catalyse l'AA en PGH_2. Ce dernier est ensuite catalysé par le TxA_2 synthase en

TxA_2 qui induit l'agrégation plaquettaire en liant les récepteurs TP. L'ASA inhibe préférentiellement la COX-1 avec une affinité qui est environ 170 fois supérieure à celle de la COX-2 (381). Ainsi, une administration de faibles doses d'ASA de 75-100 mg est capable d'inhiber la COX-1 plaquettaire et la génération de TxA_2 qui se reflète par un effet antiplaquettaire et affecte moins la COX-2 vasculaire (382, 383). Cependant, l'utilisation régulière de l'ASA peut augmenter les risques d'hémorragie, et ce à partir de faibles doses. Également, il peut augmenter l'occurrence de complications gastro-duodénales (384-386). De plus, l'ASA est capable d'inhiber la COX-1 des mégacaryocytes favorisant ainsi la production de plaquettes avec la COX-1 déjà inhibée (387). Enfin, les AINS comme l'ibuprofène et le naproxène sont aussi des inhibiteurs de la COX. Cependant, puisque la liaison est réversible, leur effet anti-thrombotique est transitoire et inapte à fournir la protection chez les patients souffrants de coronaropathies. De plus, l'interaction de l'AINS à la COX-1 prévient l'ASA d'acétyler la sérine de la COX-1 par encombrement stérique. Dès lors, la prise de l'AINS et de l'ASA devrait être écartée chez les patients souffrant de coronaropathie puisque cela pourrait augmenter le risque d'occurrence d'évènements thrombotiques (388-390) (Figure 4.2).

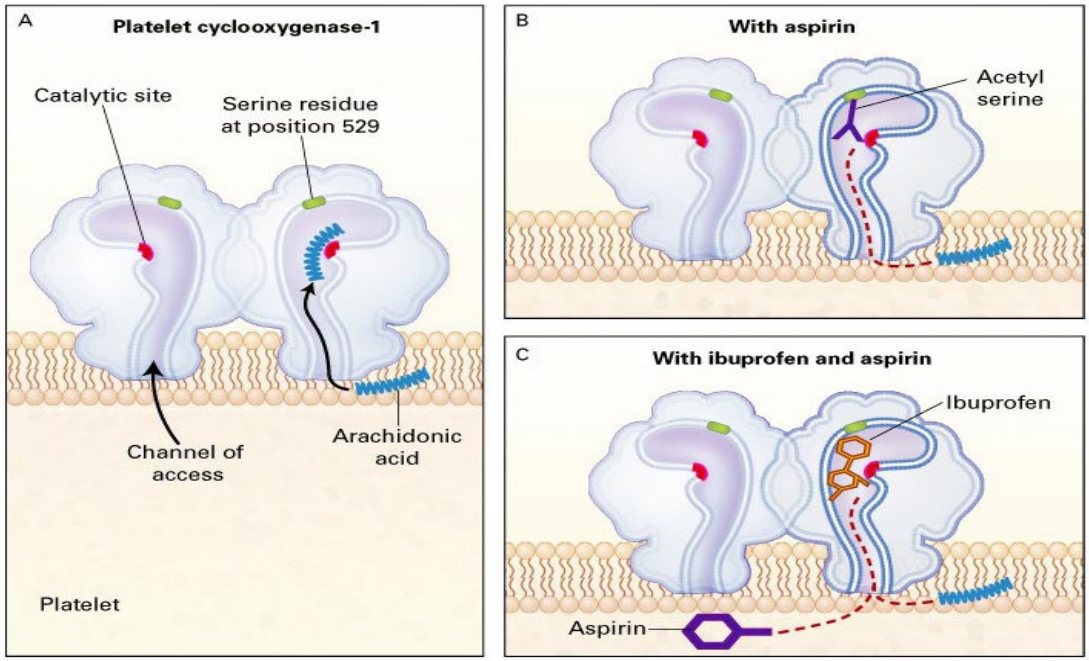

Figure 4.2: Le mécanisme d'action des inhibiteurs de COX-1. Catella-Lawson F et al. *N Engl J Med.* 2001 Dec 20;345(25):1809-17.

4.2.2 Pharmacocinétique

L'ASA est rapidement absorbé par diffusion passive au niveau gastrique et duodénal après administration orale. Il est hydrolysé en acide salicylique actif au niveau de la muqueuse intestinale, du foie et du sang. Il atteint un pic plasmatique 30 à 40 minutes après ingestion avec une biodisponibilité de 40 à 50%. Son effet antiplaquettaire TxA_2-dépendant est surtout atteint après 1h (391-393). Il est important de mentionner que les formulations comprenant un enrobage de protection gastrique ont une plus longue absorption qui est limitée, une plus faible biodisponibilité et un pic plasmatique d'environ 3-4h après ingestion (394, 395). L'ASA possède une demi-vie de 15 à 20 minutes dû à son hydrolyse rapide; cependant, malgré que la demi-vie soit particulièrement courte, l'inhibition de la fonction proagrégante des plaquettes dure tout au long de la durée de vie des plaquettes qui est d'environ de 7 à 10 jours (396-398). En revanche, puisqu'environ 10% des plaquettes sont régénérées dans la circulation quotidiennement, une dose d'ASA maintient un effet antiplaquettaire pendant environ deux jours (399). Puisque la majorité de l'ASA est rapidement hydrolysée, juste 1% de la dose administrée est excrétée de façon intacte dans l'urine. Le reste est excrété de façon rénale sous forme de métabolites inactifs comme l'acide salicylique libre, l'acide salicylurique et la glucuronide phénolique (397, 398).

4.2.3 La résistance à l'ASA

L'efficacité clinique de l'ASA à réduire le risque des évènements cardiovasculaires est bien établie. Cependant, un large nombre de patients ne bénéficie pas de l'ASA et souffre toujours d'événements thrombotiques récurrents malgré qu'ils soient sous traitement à l'ASA. Ces patients sont ainsi décrits comme étant résistant à l'ASA. En effet, il est estimé que 0.4% à 60% des patients souffrant de coronaropathie ont une réponse inadéquate à l'ASA associée à une résistance à l'ASA (400-406). Cette grande variabilité interindividuelle de réponse à l'ASA est multifactorielle; effectivement, plusieurs mécanismes contribuent au phénomène de la résistance à l'ASA (Figure 4.3).

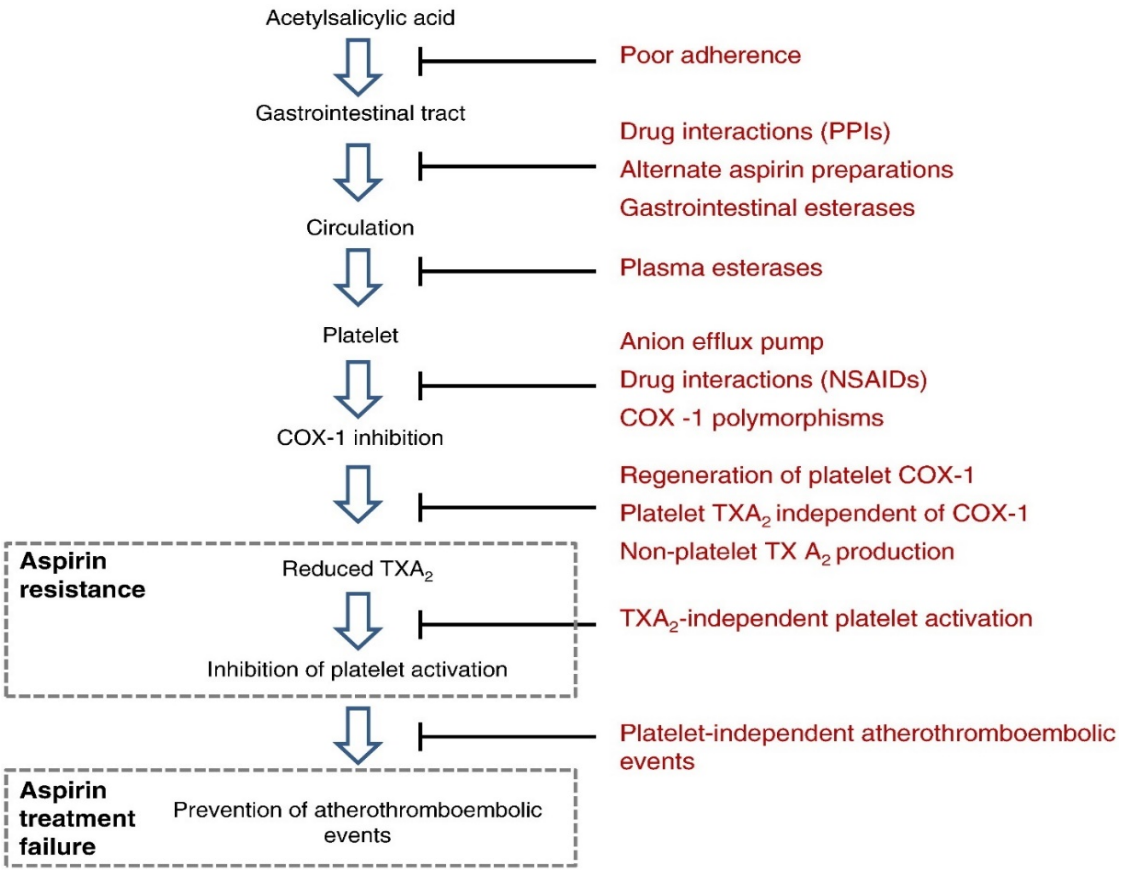

Figure 4.3: Les mécanismes de résistance à l'ASA. Floyd CN et al. Pharmacol Ther. 2014 Jan;141(1):69-78.

D'abord, une diminution de la biodisponibilité de l'ASA est un important aspect de la résistance à l'ASA. Les causes de cette diminution peuvent survenir suite à une mauvaise compliance au traitement (407, 408), une absorption réduite de l'ASA (la prise des inhibiteurs de pompes à protons (409), l'expression des transporteurs d'efflux comme la MRP4 (*multidrug resistance protein 4*) (410, 411) et la préparation de l'ASA avec un enrobage entérique (412)) et une augmentation du métabolisme de l'ASA médié par les estérases (413, 414). Ensuite, la résistance à l'ASA peut être engendrée par un défaut de liaison de l'ASA au COX-1 par interaction médicamenteuse suite à la prise de AINS (Figure 4.2), la biosynthèse de TxA_2 par des voies qui ne sont pas bloquées par l'ASA (exemple : la COX-2 des monocytes, macrophages et cellules endothéliales)(415-418) et la régénération de la COX-1 (la synthèse de novo de la COX-1 par les plaquettes ou une surproduction plaquettaire au sein de la moelle osseuse) (419, 420). De plus, des polymorphismes génétiques affectant différentes protéines dont les COX, la

TxA_2 synthase, les récepteurs plaquettaires incluant $\alpha_{IIb}\beta 3$, $\alpha_2\beta_1$, GPIb-IX-V, et le Facteur XIII peuvent participer à la résistance à l'ASA (421-425). Par la suite, un phénomène de tachyphylaxie qui résulte par une perte d'effet antiplaquettaire de l'ASA suite à son administration prolongée chez les patients est une cause possible de la résistance (426, 427). Enfin, la résistance à l'ASA peut être provoquée par des voies d'activation plaquettaire qui ne sont pas inhibées par l'ASA (exemples : stimulation par la thrombine, ADP et épinephrine)(428), ou encore, par la présence de niveaux élevés d'amorceurs circulants ou «*prime*rs» qui engendrent l'hyperactivité plaquettaire (429).

4.3 Les antagonistes de P_2Y_{12}

Le récepteur P_2Y_{12} joue un rôle crucial dans l'activation des plaquettes par l'ADP capable d'induire la signalisation «*inside-out*» du $\alpha_{IIb}\beta_3$ et l'amplification de l'agrégation plaquettaire (430). Ainsi, les antagonistes de P_2Y_{12} agissent en inhibant ce mécanisme d'amplification par l'ADP d'où leur effet antithrombotique. La double thérapie combinant l'ASA et un antagoniste de P_2Y_{12} est la pierre angulaire et «*gold standard*» pour le traitement des patients souffrant de SCA et ayant subi une ICP (431, 432). En effet, l'effet synergique de la double thérapie a été étudié dans une multitude d'essais cliniques afin d'établir le régime antiplaquettaire idéal chez les patients ayant subi une ICP (433-436) Les antagonistes de $P2Y_{12}$ approuvés comprennent deux classes: les thiénopyridines dont la ticlopidine, le clopidogrel; et le prasugrel et les non thiénopyridines dont le ticagrelor et le cangrelor.

4.3.1 La classe des thiénopyridines

Les thiénopyridines sont des prodrogues administrés oralement qui sont convertis par le Cytochrome P450 (CYP450) hépatique en métabolites actifs capables d'inhiber de façon irréversible les récepteurs de $P2Y_{12}$. La ticlopidine, le premier inhibiteur de la $P2Y_{12}$, représente la première génération des thiénopyridines approuvés par la FDA (*Food and Drug Administration*)(437, 438). Cependant, dus à ses effets indésirables fréquents d'ordre hématologique ou hémorragique (439), il a été remplacé par le clopidogrel, appartenant à la seconde génération de thiénopyridines, qui a un profil pharmacologique plus sécuritaire. Suite à son absorption, 85% du clopidogrel est hydrolysé par les estérases; il est intéressant de

mentionner que ces enzymes ont une activité plus élevée chez les diabétiques, ce qui diminue l'efficacité du médicament (440). Les 15% restants sont rapidement oxydés par le CYP450 (CYP3A4, CYP3A5, CYP2C19) en deux étapes; la première transformation est en 2-oxo-clopidogrel; ensuite en composé thiol actif et très instable, d'une demi-vie d'environ 60 minutes. Ainsi, ce métabolite actif inhibe irréversiblement le récepteur $P2Y_{12}$ de façon covalente pour toute la durée de vie des plaquettes (441). Dès lors, le clopidogrel demeure l'inhibiteur de $P2Y_{12}$ le plus utilisé et recommandé chez les patients souffrant de coronaropathie et ayant subi une ICP (442, 443). De plus, plusieurs études cliniques démontrent le bénéfice de la double thérapie de l'ASA avec le clopidogrel chez les patients souffrant de SCA et ceux qui subissent une ICP (432, 444-446). Cependant, 10% des patients recevant une double thérapie avec l'ASA et le clopidogrel souffrent d'évènements ischémiques récurrents pendant les 12 mois de thérapies double (444). De plus, 30 à 40% des patients traités avec du clopidogrel présentent une réactivité plaquettaire élevée (447-449). Cet inconvénient est attribué à la large variabilité interindividuelle de réponse au clopidogrel. La variabilité de réponse est plurifactorielle et peut être causée par un défaut dans l'absorption ou le métabolisme du clopidogrel incluant les polymorphismes génétiques des CYP450 et les interactions médicamenteuses(450-454). Ainsi, le développement d'une troisième génération de thiénopyridine, le prasugrel, présente moins de variabilité interindividuelle au niveau de sa réponse; ainsi, il y a une meilleure biodisponibilité in *vivo* et effet antiplaquettaire comparé au clopidogrel (455). Effectivement, le prasugrel ne requiert qu'une seule étape d'oxydation au niveau du CYP450 hépatique pour générer son métabolite actif (R-138727) (371, 456, 457). Des essais cliniques comme TRITON-TIMI 38 ont permis de recommander le prasugrel chez les patients souffrant de SCA qui subissent des ICP (458-462). Cependant, puisque le prasugrel a un effet antiplaquettaire puissant, il est contre-indiqué chez les patients à haut risque de saignement; ainsi, il doit être arrêté 7 à 10 jours avant toute opération afin de réduire les risques de saignement puisque le prasugrel inhibe le $P2Y_{12}$ de manière irréversible (463-466).

4.3.2 La classe des non thiénopyridines

Les non thiénopyridines sont des dérivés qui lient directement le récepteur du $P2Y_{12}$ qui présentent deux avantages en comparaison aux thiénopyridines : premièrement, elles ne requièrent pas d'activation métabolique, deuxièmement leur liaison aux récepteurs du $P2Y_{12}$ est

réversible, favorisant le rétablissement rapide de la fonction plaquettaire après l'arrêt de médication (467). Le ticagrelor est le premier cyclopentyl-triazolo-pyrimidine administré oralement, utilisé pour inhiber directement et de manière réversible le $P2Y_{12}$ en liant un site allostérique. Effectivement, au contraire des thiénopyridines qui bloquent le site de liaison de l'ADP au $P2Y_{12}$, le ticagrelor inhibe la liaison de l'ADP de façon non compétitive, qui suggère un site de liaison indépendant pour les cyclopentyl-triazolo-pyrimidines (468). Malgré qu'il ne nécessite pas d'être métabolisé par le CYP450 pour être capable d'agir, environ 30 à 40% de l'effet antiplaquettaire du ticagrelor est attribué à son métabolite actif (AR-C124910XX) qui est produit par le CYP450 (CYP3A4 et CYP3A5). Ainsi, le ticagrelor a un effet antiplaquettaire plus rapide que le clopidogrel ainsi qu'un rétablissement de la fonction plaquettaire qui se fait en 3 jours suite à la cessation de ticagrelor (469). De plus, le ticagrelor a une meilleure efficacité clinique comparée au clopidogrel, et ce selon l'étude PLATO qui a rapporté que chez les patients ayant subi un SCA, le ticagrelor induit une diminution significative du risque d'IM, d'AVC et de la mortalité cardiovasculaire par rapport au clopidogrel (470). Cependant le ticagrelor présente des effets indésirables comme la dyspnée et une altération du rythme cardiaque; ainsi, le nombre de sujets randomisés pendant l'étude PLATO a discontinué le traitement au ticagrelor était plus élevé que ceux traités avec le clopidogrel (470). Un autre inhibiteur direct et réversible du $P2Y_{12}$ est le cangrelor, qui se distingue des autres antagonistes de $P2Y_{12}$ par son administration qui est intraveineuse. Cette méthode lui procure ainsi un avantage comparé aux autres antagonistes (471). Effectivement, malgré que le prasugrel et le ticagrelor présentent une meilleure efficacité clinique que le clopidogrel, ces trois possèdent des limitations communes attribuées à leur administration orale. Ces limitations comprennent les variabilités pharmacodynamiques, un lent rétablissement de la fonction plaquettaire et la difficulté d'être administré à des patients présentant une instabilité hémodynamique, sous sédation, intubés ou souffrant de nausées et vomissement (371). Ainsi, le cangrelor se distingue par son délai d'action très rapide qui se traduit par une inhibition maximale des plaquettes dans un temps compris entre 15 à 30 min. De plus, le cangrelor (AR-C69931MX) possède particulièrement une très courte demi-vie de 3 à 6 min permettant le rétablissement de la fonction plaquettaire après 30 min à 1h suivant la cessation de l'infusion intraveineuse(472-474). Ce profile pharmacologique peut avoir un rôle avantageux chez les patients qui requiert l'administration d'un antagoniste du récepteur $P2Y_{12,}$ mais pour une durée transitoire en vue d'une chirurgie. D'ailleurs, le cangrelor

est utilisé à des doses réduites pour substituer les antagonistes de $P2Y_{12}$ oraux lorsqu'une chirurgie cardiaque est anticipée (475). Enfin, le cangrelor est approuvé pour utilisation durant une ICP afin de réduire les évènements ischémiques associés à l'emplacement d'une nouvelle endoprothèse cardiaque (476, 477).

4.4 L'antagoniste de PAR-1, le vorapaxar

La thrombine contribue à l'hémostase de différentes manières incluant l'activation plaquettaire, l'activation de la protéine C et la conversion du fibrinogène en fibrine. La thrombine est l'activateur plaquettaire le plus puissant en circulation et ces effets sont médiés surtout par le PAR-1 se trouvant à la surface des plaquettes (93, 478). Ainsi, le vorapaxar (SCH50348) est le premier antagoniste sélectif approuvé de PAR-1 administré par voie orale. Le vorapaxar est rapidement absorbé avec une grande biodisponibilité et il est métabolisé par le CYP3A4 qui résulte en un métabolite actif aussi puissant que le composé mère (129, 479). Chez les patients souffrant de SCA sans surélévation du segment ST, le vorapaxar atteint une inhibition quasi maximale des plaquettes en 2h; de plus, il possède une longue demi-vie de 173 à 269h (480). Deux grands essais cliniques de phase III, TRACER et TRA 2P-TIMI ont permis d'approuver le vorapaxar pour la réduction des évènements thrombotiques cardiovasculaires chez les patients avec historique d'IM et de maladie artérielle périphérique (MAP) (481-483). Cependant, il est contre-indiqué chez les patients avec historique d'AVC, d'accident ischémique transitoire (AIT) ou d'hémorragie intracrânienne et chez les patients présentant un saignement pathologique actif (480, 484).

4.5 Les antagonistes de l'intégrine $\alpha_{IIb}\beta_3$

La liaison du fibrinogène aux récepteurs $\alpha_{IIb}\beta_3$ constitue la phase finale et cruciale de l'agrégation plaquettaire. Ainsi, les antagonistes des récepteurs de $\alpha_{IIb}\beta_3$ (abciximab, eptifibatide et tirofiban) empêchent la liaison du fibrinogène et VWF à l'intégrine $\alpha_{IIb}\beta_3$ afin d'induire leur effet antiplaquettaire (485). Ils sont administrés par voie intraveineuse et leur effet antiplaquettaire est d'un délai d'action rapide de 10 à 30 min. L'abciximab bloque le récepteur $\alpha_{IIb}\beta_3$ de manière irréversible; ainsi, il possède une longue demi-vie de 23h. Or, le tirofiban et l'eptifibatide ont une inhibition de type compétitif réversible avec une durée de vie plus courte

de 2 et 2.5h respectivement. Le rétablissement de la fonction plaquettaire a lieu après 6h suivant la cessation du tirofiban, 8h après l'arrêt de l'eptifibatide et 72h après l'arrêt de l'abciximab (486-489). Les antagonistes des récepteurs de $\alpha_{IIb}\beta_3$ sont indiqués pour la prévention des événements thrombotiques chez les patients souffrant de SCA ayant subi une ICP et sont souvent prescrits 24 à 48h après une ICP avec pose d'endoprothèse (490). De plus, ils sont utilisés chez les patients souffrant de SCA à haut risque d'évènement thrombotique incluant les diabétiques et les patients avec le taux de troponines très élevé. Cependant, leur utilisation est limitée pour le risque élevé de saignement associé aux antagonistes des récepteurs de $\alpha_{IIb}\beta_3$ (491, 492).

Mise en contexte, hypothèse et objectifs

La présence de médiateurs thrombo-inflammatoires dans la circulation sanguine des patients atteints de maladies cardiovasculaires amplifie l'athérosclérose et la thrombose, ainsi que leurs complications cliniques ultérieures, telles que la cardiopathie ischémique, le SCA, l'IM les accidents cérébro-vasculaires (493). Le sCD40L figure parmi les principaux médiateurs thrombo-inflammatoires apparus comme prédicteur fiable des maladies cardiovasculaires (213, 267, 494), en particulier chez les patients atteints de diabète (269, 287, 288), d'hypercholestérolémie (289, 290) d'athérosclérose (291) et de SCA (270, 292, 293).

Nous avons démontré que le sCD40L lui-même n'affecte pas la fonction plaquettaire, mais stimule et potentialise l'activation plaquettaire, l'agrégation et la formation de thrombus en réponse à un stimulus thrombotique (294, 295). En effet, notre laboratoire a démontré que le sCD40L augmente la réactivité plaquettaire et amplifie les réactions thrombotiques impliquant son récepteur principal le CD40 et en liant une protéine adaptatrice, le TRAF2 et la signalisation en aval via Rac1/p38-MAPK (294).Nous avons également révélé que le sCD40L était un puissant activateur de NF-κB (295). Plus récemment, notre laboratoire a aussi révélé dans des études préliminaires que l'ASA n'affectait pas l'action d'amorçage du sCD40L sur les plaquettes, ce qui suggère que le sCD40L réduit l'efficacité des traitements antiplaquettaires.

Je postule que le ciblage de l'action d'amorçage de l'axe sCD40L/CD40 sur les plaquettes pourrait représenter un nouveau traitement préventif chez les patients à haut risque présentant des taux circulants élevés de sCD40L; et aussi la gestion clinique des patients coronariens présentant des événements athéro-thrombotiques non liés au traitement conventionnel. Ainsi dans le cadre de nos études sur l'axe sCD40L/CD40 plaquettaire, ce projet de doctorat vise spécifiquement à valider l'hypothèse principale que le sCD40L active le NF-κB via CD40 et TAK1 et l'ASA affecte la fonction du sCD40L sur les plaquettes.

Les objectifs spécifiques sont :

1) Identifier les récepteurs plaquettaires de sCD40L impliqués dans l'activation du NF-κB

2) Déterminer l'impact de TAK1 dans l'axe sCD40L/NF-κB plaquettaire

3) Déterminer l'effet de l'ASA sur l'action d'amorçage du sCD40L sur les plaquettes

Chapitre 5 : Contributions scientifiques

5.1 Premier Article: Publié dans Journal of American Heart Association en Décembre 2018

CD40L priming of platelets via NF-κB activation is CD40- and TAK1-dependent

Résumé

Mise en contexte: Le CD40 ligand (CD40L) est une molécule thrombo-inflammatoire qui prédit les événements cardiovasculaires. Le CD40L est un puissant activateur de NF-κB dans les plaquettes qui amorce et potentialise l'activation plaquettaire en réponse à des stimuli thrombotiques. En plus de son récepteur classique CD40, le CD40L se lie à $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ et $\alpha_M\beta_2$ dans divers types de cellules. Cependant, la fonction des différents récepteurs de CD40L sur les plaquettes reste inexplorée. Cette étude vise à identifier les récepteurs de CD40L, impliqués dans l'activation du NF-κB plaquettaire, leur signalisation en aval et leur implication dans l'agrégation plaquettaire.

Méthodes et Résultats: Nous avons démontré que les plaquettes exprimaient CD40, $\alpha_{IIb}\beta_3$ et $\alpha_5\beta_1$ et libéraient CD40L en réponse à une stimulation avec le sCD40L. Le sCD40L seul a induit l'activation du NF-κB plaquettaire de manière dose-dépendante. Cet effet était absent chez les plaquettes de souris $CD40^{-/-}$ et inhibé par le blocage de CD40, mais n'était pas affecté par le blocage d'$\alpha_{IIb}\beta_3$ et $\alpha_5\beta_1$ dans les plaquettes humaines. L'axe sCD40L/CD40 active TAK1 en amont de NF-κB. Dans les études fonctionnelles, le sCD40L seul n'a pas d'effet sur l'agrégation plaquettaire mais potentialise l'agrégation en présence de doses sous-optimales de thrombine; cet effet a été aboli par les inhibiteurs de CD40, TAK1 et NF-κB.

Conclusion: Le CD40L amorce les plaquettes *via* les voies de signalisation impliquant CD40/TAK1/NF-κB, ce qui prédispose les plaquettes à une activation et une agrégation prononcée en réponse à des stimuli thrombotiques.

Contribution des auteurs

Kevin Kojok: Planification et exécution des expériences d'agrégation plaquettaire et de signalisation intracellulaire (Figures 2,3 et 5-9). Analyse des résultats et montage des figures. Rédaction et correction de l'article.

Souhad El-Akoum: Planification et exécution des expériences de cytométrie en flux (Figure 1) et de signalisation intracellulaire (Figure 4), ainsi que correction de l'article.

Mira Mohsen: Participation aux techniques liées à l'agrégation plaquettaire

Walid Mourad: Codirection générale

Yahye Merhi: Direction générale et correction de l'article.

CD40L priming of platelets via NF-κB activation is CD40- and TAK1-dependent

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Running Title: CD40L/CD40/TAK1/NF-κB axis in platelet function

Journal Subject Terms: Thrombosis, Coronary Artery Disease

Background—CD40 ligand (CD40L) is a thrombo-inflammatory molecule that predicts cardiovascular events. CD40L is a strong activator of NF-κB in platelets that primes and enhances platelet activation in response to thrombotic stimuli. In addition to its classical receptor CD40, CD40L binds αIIbβ3, α5β1 and αMβ2 in various cell types. However, the function of the different CD40L receptors on platelets remains unexplored. The present study aims to identify

the receptors of CD40L, involved in platelet NF-κB activation, their downstream signalling and their implication in platelet aggregation.

Methods and Results—We showed that platelets express CD40, αIIbβ3 and α5β1 and release CD40L in response to sCD40L stimulation. sCD40L alone dose-dependently induced platelet NF-κB activation; this effect was absent in $CD40^{-/-}$ mouse platelets and inhibited by the CD40 blockade, but was unaffected by the αIIbβ3 or α5β1 blockade in human platelets. sCD40L/CD40 axis activates TAK1 upstream of NF-κB. In functional studies, sCD40L alone did not affect platelet aggregation but potentiated the aggregation response in the presence of suboptimal doses of thrombin; this effect was abolished by CD40, TAK1 and NF-κB inhibitors.

Conclusions—CD40L primes platelets via signalling pathways involving CD40/TAK1/NF-κB, which predisposes platelets to enhanced activation and aggregation in response to thrombotic stimuli.

Key Words: Platelets • NF-κB • CD40L • CD40 • TAK1

Clinical Perspective

What is new?

- In platelets, activation of NF-κB by sCD40L is CD40-dependent.
- sCD40L/CD40 axis activates TAK1 upstream of NF-κB in platelets.
- CD40L/CD40/TAK1/NF-κB signaling primes platelets and potentiates aggregation in response to thrombotic stimuli.

What are the clinical implications?

- Targeting CD40L/CD40/TAK1/NF-κB limits platelet priming and activation and may ultimately represent a therapeutic target in the treatment of thrombo-inflammatory diseases.

Introduction

Patients with inflammatory diseases exhibit higher than expected rates of ischemic cardiovascular events that are not attributable to common risk factors, but rather to inflammatory stimuli that trigger pathways contributing to the pathogenesis of cardiovascular diseases[1, 2]. In this regard, the thrombo-inflammatory mediator CD40L and its classical receptor CD40, molecules of critical importance in humoral immunity, have gained significant attention for their involvement in the pathophysiology of cardiovascular diseases and thrombotic events[3-5]. Indeed, CD40L/CD40 interactions in different cell types regulate a plethora of inflammatory cascades (cytokine release, up-regulation of adhesion molecules and activation of leukocytes and platelets) at the forefront of cardiovascular alterations. Thus, CD40L/CD40 axis may represent a pivotal contributor to the establishment of accelerated vascular alterations in patients with high circulating levels of CD40L. Like most members of the TNF family, CD40L can be detected in soluble form (sCD40L) in the blood circulation, whose principal source is activated platelets[6-8]. Circulating levels of sCD40L in patients have now emerged as reliable indicators of cardiovascular risk, as there appears to be a significant correlation between levels of sCD40L and vascular complications such as atherosclerosis and acute coronary syndromes[9, 10]. sCD40L activates platelets, as revealed by α- and dense-granule release, and morphological changes typically associated with activation of αIIbβ3[11]. In this regard, we have shown that enhanced levels of sCD40L exacerbate platelet activation and aggregation through the CD40/TRAF-2/Rac-1/p38 MAPK signalling pathways[12]. More recently, we have also shown that sCD40L triggers nuclear factor kappa B (NF-κB) activation in platelets[13]. Activation of NF-κB by sCD40L is independent of p38 MAPK, which suggests that CD40L activates NF-κB through a different pathway.

In nucleated cells, CD40L-induced NF-κB activation involves the phosphorylation of IκB (NF-κB inhibitor κB protein), thus causing its proteasome-mediated degradation. In response to stimuli, the NF-κB dimers, in association with the inhibitory IκB subunit, are regulated by the IκB kinase (IKK). Upon phosphorylation by IKK, the IκB subunit is targeted for proteasome degradation, thereby releasing an active form of NF-κB that translocates into the nucleus to transcript targeted genes[14]. In anucleated platelets, phosphorylation of IκBα, which is indicative of NF-κB activation, is also observed following platelet activation[15], and NF-κB

inhibitors lead to impairment of platelet function[16]. Indeed, inhibition of platelet NF-κB activation in response to sCD40L stimulation abolished the potentiation of platelet aggregation in response to suboptimal doses of agonists[13]. This led us to hypothesize that CD40L/NF-κB signalling in platelets plays a non-genomic role as a primer that predisposes platelets to enhanced activation and aggregation and may represent an important target against atherothrombosis.

Nevertheless, the discovery of new CD40L receptors (αIIbβ3, α5β1 and αMβ2)[17-19], in addition to its classical receptor CD40, adds complexity to the diverse interplays in which CD40L takes part in platelet function[5]. Several studies showed that CD40L-induced platelet activation passes through CD40[12, 20-22], while others examined the role of CD40L/αIIbβ3 interaction in platelet physiology[17, 23]; one study reported a role for CD40L/α5β1 interactions in platelet activity[24]. Despite these studies, the contribution of each of these different receptors in platelet activation and aggregation in response to CD40L, and in particular in the activation of the NF-κB cascade, remains unexplored.

In search of the effector that leads to sCD40L-induced NF-κB activation, the transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1)[25] appeared as a principal candidate. In fact, TAK1 has been shown to function upstream of IKKβ in response to various NF-κB-inducing stimuli[26, 27]. Indeed, TAK1 is a major regulator of the inflammatory and immunity signalling pathways that can be activated through a variety of pro-inflammatory receptors, such as CD40[28]. Once activated, TAK1 leads to downstream activation of several pathways, including NF-κB[26, 29]. Although TAK1 is present in platelets[30], its function in the CD40L/NF-κB signalling remains unknown.

Thus, the present study was conducted to identify the CD40L receptors involved in platelet NF-κB activation, their downstream signalling and their impact on platelet aggregation. We found that sCD40L triggers activation of NF-κB and primes platelets through the CD40 receptor and activation of TAK1, which suggests that CD40L primes platelets via signalling pathways involving CD40/TAK1/NF-κB activation that predisposes platelets to enhanced activation and aggregation in response to thrombotic stimuli.

Methods

The data, analytic methods, and study materials will be made available, upon request from the corresponding author, to other researchers for the purpose of reproducing the results or replicating the procedure.

Preparation of human platelets

Venous blood was drawn from 30 healthy volunteers who had not taken medications known to interfere with platelet function for at least three weeks before the experiment. The protocol was approved by the Human Ethical Committee of the Montreal Heart Institute in accordance with the Declaration of Helsinki for experiments involving humans. Informed consent was obtained from all participants. Washed platelets were prepared as previously described [12, 13]. Briefly, platelet-rich plasma (PRP) was obtained by centrifugation of acid-citrate-dextrose, anti-coagulated blood. Platelets were then pelleted from PRP, to which 1 μg/mL of PGE_1 was added, washed with HBSS-Hank's sodium citrate buffer and finally resuspended in HBSS-Hank's buffer, which contained 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Platelets were adjusted to 250 $x10^6$ /mL and allowed to rest at 37ºC for at least 30 minutes before further manipulation.

Preparation of murine platelets

The handling and care of mice complied with the guidelines established by the Animal Care and Ethical Committee of the CHUM research Centre in agreement with the Canadian Council on Animal Care guidelines. Age- and sex-matched 18 wild-type (WT) and 18 $CD40^{-/-}$ mice, both on C57BLK/J6 background, were purchased from the Jackson Laboratory and housed under pathogen-free conditions. Briefly[12], mice (3-4 months; 20-30 g) were anesthetized using an intra-peritoneal injection of a mixture of 75 mg/kg Ketamine (Vetalar) and 0.5 mg/kg Medetomidine (Domitor, Pfizer). Blood was collected from the ventricular puncture into syringes containing one-twentieth of the blood volume of heparin as an anticoagulant. For each experiment, we combined the blood collected from three mice (750-1,000 μL/mouse), which

was diluted (1:1) using a modified Tyrode buffer containing 0.1 μg/mL PGE_1 and centrifuged. Washed platelets were then prepared from PRP and resuspended in a modified Tyrode buffer to yield a final concentration of 250 x 10^6 /mL.

Determination of platelet CD40L receptors

The known CD40L receptors in various cell types are CD40, αIIbβ3, α5β1 and αMβ2. Their expressions on human platelets were first measured by flow cytometry. Isolated platelets were fixed with 1% paraformaldehyde and were then washed and stained with saturating concentrations of primary antibody against CD40, α5, β1, αIIb, β3, αM and β2 integrins (R&D systems) for 30 minutes at 4ºC or their isotype-matched control IgGs. Platelets were washed three times with PBS/0.2% FBS, and Alexa 488 coupled secondary antibody was then added and incubated for 30 minutes at 4ºC. Samples were analysed (20,000 events) on a FACSCalibur flow cytometer (Becton Dickinson), and platelets were gated by their characteristic forward and side scatter properties, as described previously[12].

The presence of the different CD40L receptors in platelets was also measured by western blot (WB) of platelet lysates. Proteins were resolved in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk for 1 hour, washed three times with TBS/T (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1 % Tween-20) and incubated with appropriate primary antibody overnight at 4ºC. Following the washing steps, membranes were labelled with horseradish peroxidase-conjugated secondary antibodies for 1 hour; washing and bound peroxidase activity was detected using enhanced chemiluminescence (PerkinElmer Life Sciences).

Measurement of platelet CD40L

The presence of CD40L in platelet lysates was determined by WB, and its expression on the platelet surface was analysed by flow cytometry. The concentration of sCD40L secreted by platelets in a resting state and after stimulation with sCD40L was determined by using an ELISA kit (R&D system) that recognizes the sCD40L backbone.

Platelet NF-κB and TAK1 activation assay

Following stimuli that solicit the NF-κB pathway, the IκB inhibitory subunit of the NF-κB complex is phosphorylated and degraded, thus allowing the p65 subunit to be phosphorylated and activated. As described previously[13] we used primary antibodies against phospho-IκBα serine$^{32/36}$, phospho-TAK1 threonine$^{184/187}$, phospho-NF-κB p65 serine^{536}, TAK1, NF-κB p65 and β-actin (Cell Signaling Technology). Isolated platelets were suspended in HBSS complete medium at 10^9 platelets/mL and were rested at 37°C for 30 minutes. Resting platelets (control) and platelets stimulated with sCD40L for 5 min at 37°C were prepared. To elucidate the involvement of each CD40L receptor in NF-κB activation, Reopro® Abciximab, 10 μg/mL, Janssen/Lilly)[17, 31] and JBS5 (10 μg/mL, Calbiochem)[24] were used to block αIIbβ3 and α5β1, respectively. In order to block the CD40 receptor, a direct CD40 blocker could not be used because the available anti-CD40 monoclonal antibody has agonistic features[32, 33]. Therefore, we acquired the Fab-fragment 5C8 (10 μg/mL), an anti-CD40L antagonist that specifically blocks the binding site of CD40L that is recognized by CD40, thereby inhbiting CD40/CD40L binding[18, 34]. The corresponding IgG isoforms for each antibody was used as a control. BAY 11-7082 (10 μM, selective IκBα inhibitor; Sigma-Aldrich)[13], 5Z-7-Oxozeaenol (ZOL, 1-1000 nM, a selective TAK1 inhibitor; Sigma-Aldrich)[35, 36] and Takinib (0.01-100 μM, a selective TAK1 inhibitor, MedChemExpress)[37] were used to study TAK1 activation by sCD40L. Platelets were then lysed by adding hot SDS-blue containing β-mercaptoethanol and protease inhibitors and the lysates immunoblotted, as described previously[12, 13].

Platelets isolated from WT or CD40$^{-/-}$ mice were suspended in a modified Tyrode buffer at 10^9 platelets/mL and rested at 37°C for 30 minutes. They were then stimulated with mouse sCD40L (msCD40L; R&D System) to evaluate NF-κB complex activation. Lysates from mouse platelets were resolved in 10% SDS–PAGE and assessed for p-IκBα and p-P65. β-actin blots were generated from stripped membranes of p-IκB and p-P65 blots.

Measurement of platelet aggregation

We monitored aggregation of washed human platelets on a four-channel optical aggregometer (Chronolog Corp.) under shear (1,000 rpm) at 37ºC. Platelet suspensions were pre-incubated with increasing concentrations (10-1,000 ng/mL) of sCD40L (R&D systems) at 37ºC, for 5, 15 or 30 minutes. Platelet suspensions were also pre-treated with several inhibitors: 5C8 (10 µg/mL), JBS5 (10 µg/mL), BAY 11-7082 (10 µM), ZOL (10, 50 and 100 nM) or Takinib (1, 5 and 10 µM), for 10 minutes followed by a treatment of platelets with sCD40L (1,000 ng/mL) for 30 minutes. Platelet aggregation was then triggered by a suboptimal dose of α-thrombin (Sigma-Aldrich) approximating 0.025 ± 0.01 U/mL that induces no more than 30% aggregation, as observed in the dose-response curve of platelet aggregation in response to thrombin ranging from 0.0125 to 0.1 U/mL (Supplemental Figure 1). Traces were recorded until stabilization of platelet aggregation was reached[12, 13].

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 25. Results are presented as mean ± SEM. Statistical comparisons were done using a paired t-test or a one-way ANOVA followed by a Dunnett's test for comparison against a single group. In human platelets, each experiment (n) represents data obtained from one donor blood; wheras in mouse, each experiment (n) represents data obtained from platelets isolated from combined 3 mouse blood. The specific statistical tests used, the mean of data, the number (n) of experiments and the P values are specified in the figure legends. A P value of < 0.05 was considered statistically significant.

Results

Detection of the CD40L system in platelets

First, we aimed to identify the different platelet CD40L receptors. This was done by analysing, using flow cytometry and WB, the different subunits of the known receptors for CD40L (CD40, αIIbβ3, α5β1 and αMβ2) on various cell types. As shown in Fig. 1A, we detected by flow cytometry the presence of CD40, αIIbβ3 and α5β1 on platelets; however, αMβ2, whose expression is restricted to leukocytes, was absent. This was confirmed by immunoblotting of the different subunits in platelet lysates (Fig. 1B).

We also confirmed the presence of the CD40L protein in platelet lysates through flow cytometry and WB (Fig. 1C). Moreover, we demonstrated its release by treating platelets with sCD40L. The platelet release of sCD40L was abolished by the blockade of CD40L/CD40 axis (Fig. 1C) using 5C8, a monoclonal antibody that binds with high affinity to CD40L and neutralizes its function by specifically blocking its binding site with CD40[34].

sCD40L activates platelet NF-κB via CD40

We have previously shown that NF-κB is required for sCD40L-induced platelet activation and potentiation of platelet aggregation independently of p38-MAPK activation[13]. Having shown that platelets express different receptors for CD40L, we aimed to determine the CD40L receptors involved in NF-κB activation. To this end, we first confirmed the activation of NF-κB by sCD40L by assessing IκBα phosphorylation, which leads to the degradation and release of the active form of NF-κB, and p65 phosphorylation, a subunit of NF-κB. Our results showed that sCD40L induces a dose-dependent phosphorylation of IκBα (Fig. 2A) and p65 (Fig. 2B). To confirm the specific action of sCD40L on platelet NF-κB activation, its action was abolished by a blocking monoclonal CD40L antibody (TNFSF5)[38] (Fig. 2). Moreover, sCD40L alone primes platelets via either NF-κB or p38 MAPK, independently of the presence of agonists such as thrombin or collagen, which were without any effects on NF-κB or p38 MAPK activation (Supplemental Figure 2).

To determine the CD40L receptors involved in platelet NF-κB activation, we employed specific blocking monoclonal antibodies to antagonize each receptor: Reopro® for αIIbβ3[17], JBS5 for α5β1[39], and 5C8 for CD40L/CD40 binding[34]. As shown in Fig. 3, blockade of αIIbβ3 (Fig. 3A) and α5β1 (Fig. 3B) receptors on platelets with their corresponding antagonists has no influence on IκB and p65 phosphorylation in response to sCD40L. In contrast, phosphorylation of both IκB and p65 (Fig. 3C) were abolished by 5C8, which binds with high affinity to CD40L and neutralizes its function by specifically blocking its binding site with CD40[34]. We confirmed the unique contribution of CD40 to platelet NF-κB activation in response to sCD40L using a genomic approach in CD40-deficient mouse platelets. Indeed, we demonstrated an increase in IκBα (Fig. 4A) and p65 phosphorylation (Fig. 4B) in msCD40L-treated, wild-type mouse

platelets; this effect was absent in CD40$^{-/-}$ mouse platelets. These results clearly showed that the CD40L receptor responsible for NF-κB activation in platelets is CD40.

sCD40L primes platelets and potentiates platelet aggregation via CD40

To obtain insights into the role of the sCD40L/CD40 axis in platelet function, we examined its effect on platelet aggregation. As shown previously[12], we first sought to confirm that sCD40L did not affect platelet aggregation by itself. Treatment of platelets with sCD40L alone did not affect aggregation independently of its concentration (Fig. 5A). However, pre-treatment of platelets with sCD40L for 5, 15 or 30 minutes lead to a significant and dose-dependent increase of aggregation in response to a suboptimal dose of thrombin (Fig. 5B). For example, pre-treatment of platelets with sCD40L for 30 minutes significantly potentiates the response to a suboptimal dose of thrombin from approximately 15% in the absence of sCD40L to 40%, 75% and 85% with 10, 100 and 1,000 ng/mL of sCD40L, respectively. This indicates that sCD40L primes platelets and predisposes them to increased aggregation in response to a suboptimal concentration of platelet agonists. Moreover, sCD40L potentiated platelet aggregation in the presence of a suboptimal dose of TRAP-1 replicating in a similar fashion the potentiation observed with a suboptimal dose of thrombin, indicating that the action of thrombin is related to its platelet activating property, independently from its proteolytic activity (Supplemental Figure 3).

Next, we sought to determine if such response involves CD40. Effectively, as shown in Fig. 6A, blockade of the CD40L/CD40 axis before treatment with sCD40L abolished the potentiation in platelet aggregation in response to a suboptimal dose of thrombin. In contrast, α5β1 blockade did not (Fig. 6B). Blockade of αIIbβ3 with Reopro® cannot be tested to observe the effect of sCD40L on platelet potentiation, because it is a platelet aggregation inhibitor that hinders the binding of fibrinogen to αIIbβ3 receptors, which is a necessary step for platelet aggregation. Finally, we confirmed the implication of NF-κB in platelet priming through the use of BAY 11-7082, a specific inhibitor of NF-κB, which hinders the potentiating role of sCD40L in platelet aggregation (Fig. 6C).

TAK1 is required in CD40L/CD40/NF-κB signalling and platelet priming

After demonstrating the involvement of CD40L/CD40/NF-κB signaling in platelet priming, we sought to determine whether TAK1 is the effector protein involved upstream of NF-κB. We showed that TAK1 is present in platelets and is phosphorylated upon sCD40L activation (Fig. 7). Moreover, we demonstrated that TAK1 is upstream of NF-κB because its inhibitors, ZOL and Takinib, in a dose-dependent manner, abolish IκB phosphorylation when stimulated with sCD40L. Indeed, at 100 nM, ZOL inhibits entirely TAK1 and IκB phosphorylation (Fig. 8A). Similarly, at 10 μM, Takinib suppresses TAK1 and IκB phosphorylation (Fig. 8B). Such results highlight the requirement of TAK1 for sCD40L-induced NF-κB activation in platelets. Furthermore, blockade of TAK1 with ZOL (Fig. 9A) or Takinib (Fig. 9B) dose dependently impairs the potentiating action of sCD40L on platelet aggregation, highlighting for the first time the key involvement of TAK1 in CD40L activation of NF-κB and priming of platelets. Notes that ZOL or Takinib alone have no effects on platelets aggregation (Supplemental Figure 4).

Discussion

Platelets are vital players in immune and inflammatory reactions[40, 41]. In particular, platelet CD40L, expressed on the surface of activated platelets and cleaved to generate most of the sCD40L within the blood circulation, induces inflammatory and thrombotic responses in the vascular system4, [5, 20, 42]. However, the mechanisms involved in its modulation of platelet function remain unclear. Our previous studies have shown that sCD40L alone, independently of the presence of agonists such as thrombin or collagen, primes platelets via different signaling mechanisms, which includes a CD40-dependent TRAF-2/Rac1/p38 MAPK signaling pathway[12]. In fact, the effect of sCD40L is CD40 dependent, since a mutated form of sCD40L that does not bind CD40 ($sCD40L^{R/Y}$), and $CD40^{-/-}$ mouse platelets failed to elicit such responses. More recently, our group also showed that sCD40L alone triggers a strong NF-κB activation in platelets[13]. Inhibition of IκBα reverses sCD40L-induced IκBα phosphorylation without affecting p38 MAPK phosphorylation. On the other hand, inhibition of p38 MAPK has no effect on IκBα phosphorylation, indicating a divergence in the signaling pathway originating from CD40L. However, it remains unclear if sCD40L triggers NF-κB activation via CD40 alone or with its other receptors. In addition, the downstream signaling and their impact on platelet aggregation remain unexplored.

In nucleated cells, CD40L/CD40 axis induces activation of the NF-κB signalling pathway to trigger the transcription of key genes involved in inflammation that may lead to increased cardiovascular events[43]. Although the presence of NF-κB in nucleated cells has been well documented since its discovery in immune cells over 30 years ago[44], it was not until 2002 that Liu et al. demonstrated the expression NF-κB in platelets and how thrombin-induced platelet activation triggers the degradation of IκBα[15]. Treatment with pharmacological inhibitors of NF-κB impairs platelet function, thus highlighting the non-genomic roles of NF-κB in platelet biology[16, 45]. Our group also showed that sCD40L could activate NF-κB and thus predisposes platelets to enhanced aggregation in response to a thrombotic stimulus[13]. However, CD40L has multiple receptors on platelets that might be involved in platelet function[23, 24].

We first confirmed that the priming action of sCD40L alone, via either p38MAPK or NF-κB, is insufficient to trigger platelet aggregation but potentiates platelet aggregation in response to suboptimal concentrations of platelets agonists, like thrombin or collagen, which are unable themselves to activate p38MAPK or NF-κB. Furthermore, we showed the ability of sCD40L, independently of the aggregation process, to act as a potent activator of platelet NF-κB through the phosphorylation of IκBα and p65. Such activation could be involved in platelet secretion, as we demonstrated that the main activator of NF-κB involved in platelet secretion, IKKβ, is phosphorylated upon sCD40L stimulation and its inhibition reduces the translocation of P-selectin from α granules to the platelet surface[13]. IKKβ implication on platelet secretion is related to the phosphorylation of synaptosomal-associated protein-23 (SNAP-23), which acts as a regulator of granule secretion[46]. Wei et al[47], who show that platelets with IKKβ deficiency exhibit decreased secretion and activation, confirmed this observation.

Since its discovery, CD40L was thought to possess only one receptor, CD40[48]. However, several studies have indicated the existence of additional receptors, such as αIIbβ3, α5β1 and αMβ2 or Mac-1[17, 18, 23, 49]. Of the four known CD40L receptors, CD40, α5β1 and αMβ2 are expressed on a wide variety of cell types, whereas αIIbβ3 is exclusively expressed on platelets and megakaryocytes. We first confirmed the presence, expression and release of sCD40L by activated platelets and demonstrated the presence and expression of its receptors CD40, αIIbβ3 and α5β1; however, we did not find the presence of αMβ2, which is expressed on leukocytes[50]. To elucidate whether αIIbβ3 or α5β1 triggers platelet NF-κB activation in response to CD40L, we used two relevant antagonists: Reopro® for αIIbβ3[17] and JBS5 for α5β1[39]. We observed no

changes in the phosphorylation of IκBα and p65 with or without the antagonists following sCD40L stimulation. To test the involvement of CD40, we used 5C8, which is an anti CD40L/CD40 antagonist[34]. Interestingly, NF-κB activation was abolished entirely by 5C8 following CD40L stimulation, which demonstrates that CD40L induces platelet NF-κB activation exclusively through CD40. To strengthen our observation, we confirmed those findings in a knockout mouse model, and similar to the CD40/CD40L blockade, NF-κB activation by msCD40L was absent in the $CD40^{-/-}$ mouse platelets. We had previously demonstrated that injection of msCD40L exacerbates thrombosis in WT mice but not in $CD40^{-/-}$ mice, indicating that enhanced levels of CD40L prime platelets in a CD40-dependent manner and thus predispose them to enhanced thrombus formation[12]. In the present study, we show that sCD40L triggers its intrinsic platelet secretion via CD40. In fact, minimal concentrations of sCD40L can induce significant intrinsic secretion of sCD40L from platelets compared to un-stimulated platelets; this effect is inhibited by the blockade of CD40L/CD40 binding. Moreover, blockade of CD40L/CD40 and NF-κB before treatment with sCD40L abolished the potentiation of platelet aggregation in response to a suboptimal dose of thrombin. Kuijpers et al[51], who claim that CD40 is not involved in CD40L-induced platelet priming, dispute this finding. However, several studies, including our own[11, 12, 52, 53], yield opposite results in this regard.

Finally, we demonstrated the critical role of TAK1 in platelet priming downstream of CD40L/CD40 and upstream of NF-κB. In fact, we showed the presence of TAK1 in platelets; CD40 blockade inhibits its phosphorylation upon sCD40L ligation. Moreover, two TAK1 inhibitors, ZOL and Takinib, in a dose dependent manner, abolish NF-κB activation following CD40L activation and block the priming action of CD40L on platelet aggregation. Previous studies have shown that TAK1 plays a central role in regulating proinflammatory signalling cascades in several cell types[26, 28]. The evidence for the involvement of TAK1 in CD40-induced NF-κB activation was first reported in carcinoma cells. Stimulated CD40, through its association with TRAFs, triggers the engagement of the TAK1/IKKβ/IκBα cascade, which leads to the mobilization of p65 to the IRF-1 promoter and the stimulation of IRF-1 transcriptional activation[54]. Furthermore, TAK1 mediates the production of ROS as well as the production of inflammatory mediators in a manner that implies the CD40L/CD40/NF-κB pathway in vascular smooth muscle cells[55].

We recognize that this study has some minor limitations that should be mentioned. sCD40L is released in humans in vivo in thrombotic conditions, although it attains circulating concentrations lower than those required in vitro to prime platelets. Indeed, at least 100 ng/ml of sCD40L was necessary to induce platelet NF-κB activation; whereas the levels of sCD40L in the circulating blood approximate 1-5 ng/ml and increased 10 folds up to 50 ng/ml in diabetic or in patients with thrombotic events[56, 57]. However, the level of sCD40L is predicted to be higher at the site of vascular injury. Indeed, the environment that forms in the gaps between aggregated platelets and the injured vessel wall might allow attaining active higher localized concentrations[58], than those found in the circulating blood. The study may be also limited by the fact that we did not assess the involvement of CD40L in TAK1 induced NF-κB activation and platelet priming in in vivo experimental thrombosis model, which is planned in further investigations.

In summary, the present study adds more insights on the role of CD40L in platelet function showing that 1) activation of platelet NF-κB by sCD40L is CD40-dependent; 2) sCD40L/CD40 axis activates TAK1 upstream of NF-κB; and 3) CD40L/CD40/TAK1/NF-κB signaling primes platelets and potentiates aggregation in response to thrombotic stimuli. Elevated levels of sCD40L are now considered reliable predictors of cardiovascular diseases7, particularly in patients with diabetes[56, 59], hypercholesterolemia[60] atherosclerosis[61] and ACS[10, 57, 62]. Thus, targeting CD40L/CD40/TAK1/ NF-κB limits platelet priming and activation and may ultimately represent a therapeutic target in the treatment of thrombo-inflammatory diseases.

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Disclosures

None.

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Figure legends

Figure 1. Human platelets express three receptors for CD40L: CD40, αIIbβ3 and α5β1. (**A**) CD40L receptor expression was evaluated by flow cytometry. Isolated platelets were stained with primary antibodies against CD40, α5, β1, αIIb, β3, αM and β2 integrins (grey curves), for 30 minutes at 4ºC or their isotype-matched control IgGs (black curves). The graphs of the number of platelets were normalized to the mode to depict the data regarding '% of max.' The % of max denotes the number of cells in each bin (the numerical ranges for the parameter on the *x*-axis) divided by the number of cells in the bin that contains the largest number of cells). (**B**) The presence of CD40L receptors in human platelets was also detremined by WB. Platelet lysates were resolved in 10% SDS–PAGE and assessed for CD40, α5, β1, αIIb, β3, αM and β2 integrins. (**C**) Human platelets express and release sCD40L. Left: Platelet lysates were resolved in 10% SDS–PAGE and assessed for CD40L by WB. Centre: Isolated platelets were analysed by flow cytometry using primary antibody against CD40L (grey curve) and its corresponding isotype-matched control IgG (black curve). Right: sCD40L secretion profile of platelets after stimulation with exogenous sCD40L (1 ng/mL), as analysed by ELISA. Also showing, the effect of CD40L/CD40 blockade using 5C8 on sCD40L release in CD40L-stimulated platelets (n = 3, mean ± SEM). *$p<0.01$ (Paired t-test; 5C8 vs. its IgG control).

Figure 2. sCD40L phosphorylates IκBα and P65 in human platelets. Washed human platelets (1,000 x 10^6/mL) were treated with different concentrations of sCD40L (1, 10, 100 and 1,000

ng/mL) for 5 minutes at 37°C. Platelets were also pre-treated with anti-CD40L and stimulated with sCD40L (1,000 ng/mL). Platelet lysates were resolved in 10% SDS–PAGE and assessed for (**A**) pIκBα and (**B**) pP65. Actin blot is from stripped membranes of pIκBα blot; and P65 blot is from stripped membranes of pP65 blot. Blots are representative of four independent experiments. Histograms represent the mean of data, expressed in optical density (n = 4, mean ± SEM). *p <0.05; ***p<0.001 (one-way ANOVA followed by Dunnett's multiple comparisons test *vs.* control).

Figure 3. Platelet NF-κB activation by sCD40L is CD40-dependent. Washed human platelets (1,000 x 10^6/mL) were treated with (**A**) 10 µg/mL of Reopro®, (**B**) 10 µg/mL of JBS5 or (**C**) 10 µg/mL of 5C8 and corresponding IgGs for 5 min at 37°C. Pre-treated platelets were then stimulated with 1,000 ng/mL of sCD40L for 5 minutes. Platelet lysates were resolved in 10% SDS–PAGE and assessed for pIκBα and pP65. Actin blot is from stripped membranes of pIκBα blot; and P65 blot is from stripped membranes of pP65 blot. Blots are representative of four independent experiments. Histograms represent the mean of data, expressed in optical density (n = 4, mean ± SEM). *p<0.001 (one-way ANOVA followed by Dunnett's multiple comparisons test *vs*. the sCD40L group).

Figure 4. NF-κB activation in response to msCD40L is absent in $CD40^{-/-}$ mouse platelets. Washed platelets (1,000 x 10^6/mL) from WT or $CD40^{-/-}$ mice were stimulated with different concentrations of msCD40L for 5 minutes at 37°C. Platelet lysates were resolved in 10% SDS–PAGE and assessed for (**A**) pIκBα and (**B**) pP65. Actin blot is from stripped membranes of either pIκBα blot or pP65 blot. Blots are representative of six independent experiments.

Histograms represent the mean of data, expressed in optical density (n = 6, mean ± SEM). *p< 0.05; **p<0.01 (Paired t-test; $CD40^{-/-}$ *vs.* WT).

Figure 5. sCD40L potentiates platelet aggregation in response to a suboptimal dose of thrombin. (**A**) Washed human platelets (250 x 10^6/mL) were treated with sCD40L (10, 100 and 1,000 ng/mL) for 30 minutes. Histograms represent the mean of aggregations (n = 4, Mean ± SEM). (**B**) Platelets were treated with sCD40L (10, 100 and 1,000 ng/mL) for 5, 15 and 30 minutes at 37°C. Aggregation was induced by a suboptimal dose of thrombin (0.025 ± 0.01 U/mL). Histograms represent the mean of aggregations (n = 4, mean ± SEM). *p<0.05; **p<0.01; ***p<0.001 (one-way ANOVA followed by Dunnett's multiple comparisons test *vs.* control).

Figure 6. sCD40L primes platelets via CD40 and NF-κB. Washed human platelets (250 x 10^6/mL) were pre-treated with (**A**) 10 µg/mL of Fab fragment of 5C8 and its IgG, (**B**) 10 µg/mL JBS5 or (**C**) 10 µM BAY 11-7082 for 5 minutes then stimulated with sCD40L (1,000 ng/mL) for 30 minutes at 37°C. Platelet aggregation was induced by a suboptimal dose of thrombin (0.025 ± 0.01 U/mL) in the presence or absence of sCD40L. Histograms represent means of aggregation (n = 4, mean ± SEM). *p<0.001 (one-way ANOVA followed by Dunnett's multiple comparisons test *vs*. the sCD40L group).

Figure 7. TAK1 is present in platelets and phosphorylated by sCD40L. Washed human platelets (1,000 x 10^6/mL) were treated with 10 µg/mL of blocking polyclonal anti-CD40L 37°C, then stimulated with 1,000 ng/mL of sCD40L for 5 minutes. Platelet lysates were resolved in 10% SDS–PAGE and assessed for pTAK1. TAK1 blot is from stripped membranes of pTAK1 blot.

Blots are representative of four independent experiments. Histograms represent the mean of data, expressed in optical density (n = 4, mean ± SEM). ***p<0.001 (one-way ANOVA followed by Dunnett's multiple comparisons test *vs.* control).

Figure 8. sCD40L activates TAK1/NF-κB in platelets. Washed human platelets (1,000 x 10^6/mL) were treated with several concentrations of **(A)** ZOL or **(B)** Takinib for 5 min at 37°C, and then stimulated with 1,000 ng/mL of sCD40L for 5 minutes. Platelet lysates were resolved in 10% SDS–PAGE and assessed for pIκBα and pTAK1. Actin blot is from stripped membranes of pIκBα and pTAK1. Blots are representative of four independent experiments. Histograms represent the mean of data, expressed in optical density (n = 4, mean ± SEM). For pIκBα: **p<0,001, *p<0,01 *vs.* unstimulated platelets. For pTAK1: ††p<0,001, †p<0,01 *vs.* unstimulated platelets (one-way ANOVA followed by Dunnett's multiple comparisons test *vs.* the unstimulated groups).

Figure 9. TAK1 inhibition reduces the potentiation action of sCD40L on platelet aggregation. Washed human platelets (250 x 10^6/mL) were pretreated with three doses of **(A)** ZOL (10, 50 and 100 nM) or **(B)** Takinib (1, 5 and 10 μM), then stimulated with sCD40L (1,000 ng/mL) for 30 minutes at 37°C. Platelet aggregation was induced by a suboptimal dose of thrombin (0.025 ± 0.01 U/mL). Histograms represent means of aggregation (n = 4, mean ± SEM). *p<0,01,**p<0,001 (one-way ANOVA followed by Dunnett's multiple comparisons test *vs*. the control groups without ZOL or Takinib).

Figure 1

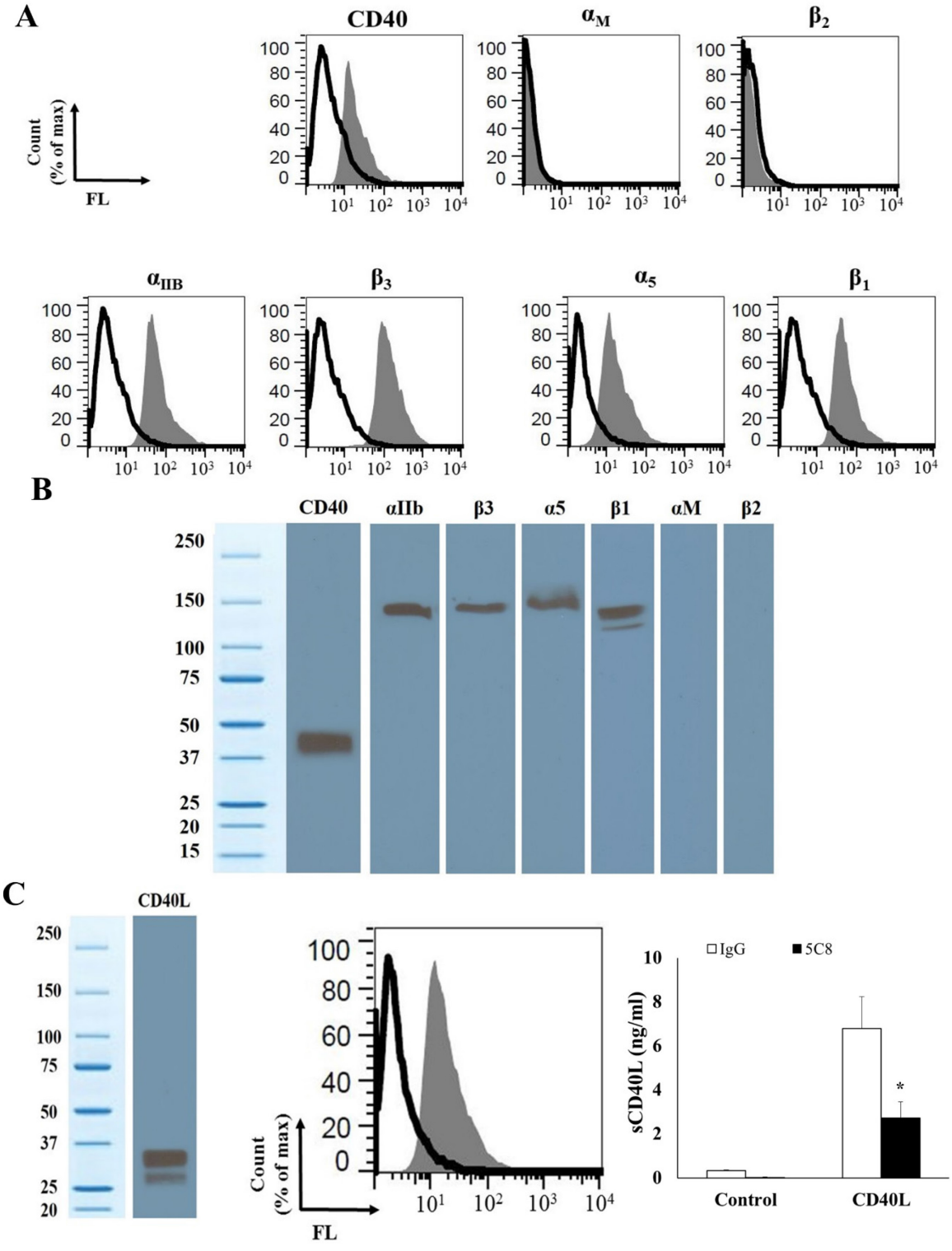

A
CD40
αM
β2
αIIB
β3
α5
β1
Count (% of max)
FL
B
CD40
αIIb
β3
α5
β1
αM
β2
250
150
100
75
50
37
25
20
15
C
CD40L
Count (% of max)
FL
IgG
5C8
sCD40L (ng/ml)
Control
CD40L
*

Figure 2

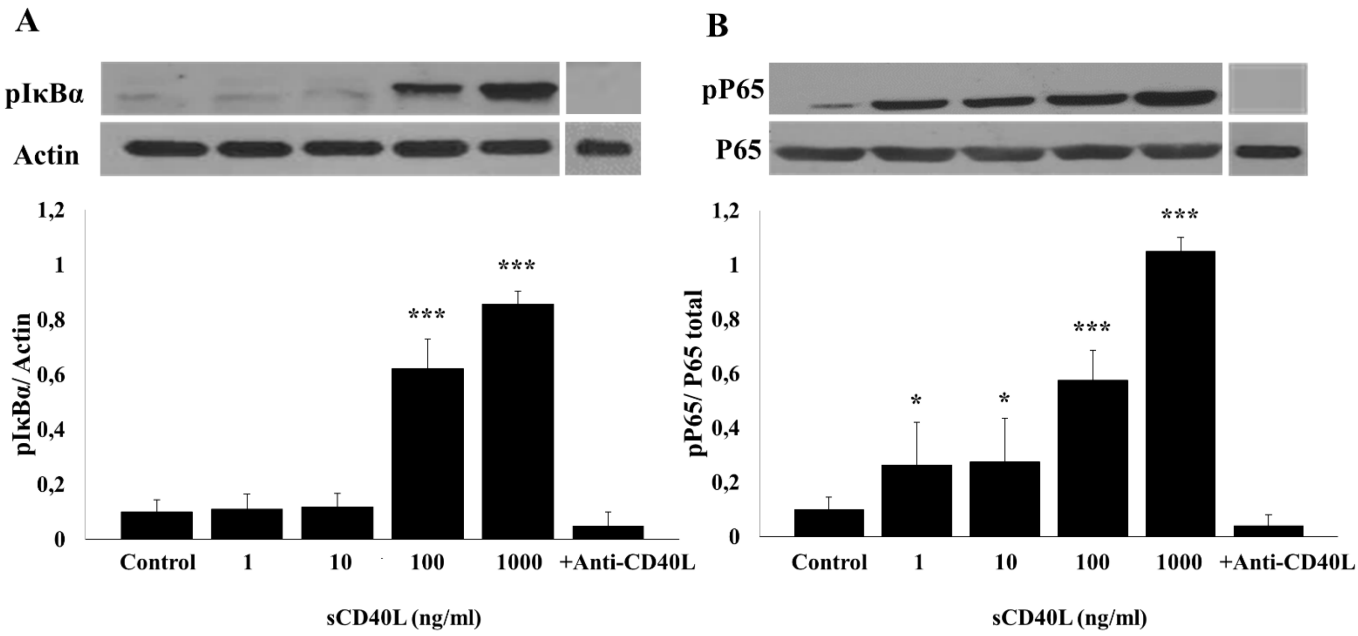

A
pIκBα
Actin
1,2
1
0,8
0,6
0,4
0,2
0
pIκBα/ Actin
***
***
Control
1
10
100
1000
+Anti-CD40L
sCD40L (ng/ml)
B
pP65
P65
1,2
1
0,8
0,6
0,4
0,2
0
pP65/ P65 total
*
*
***
***
Control
1
10
100
1000
+Anti-CD40L
sCD40L (ng/ml)

Figure 3

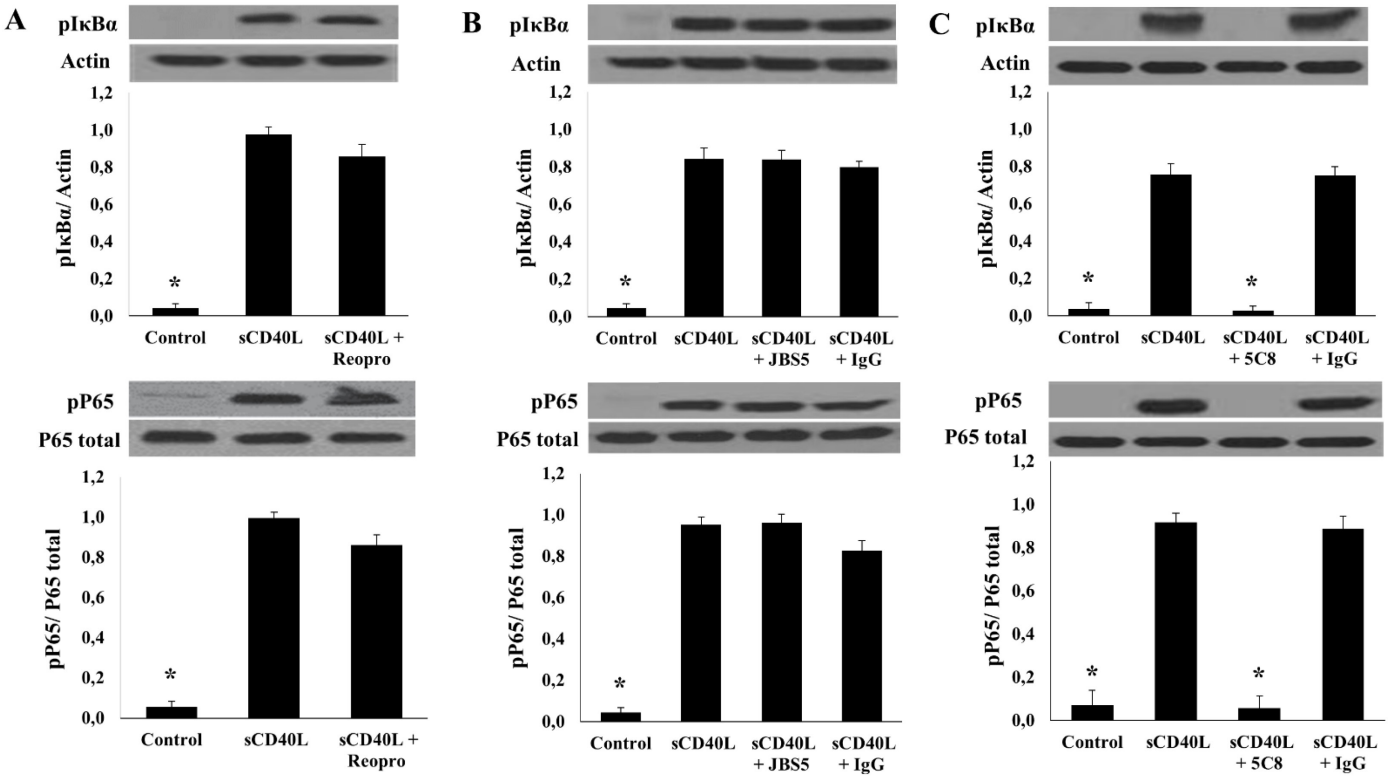


Figure 4

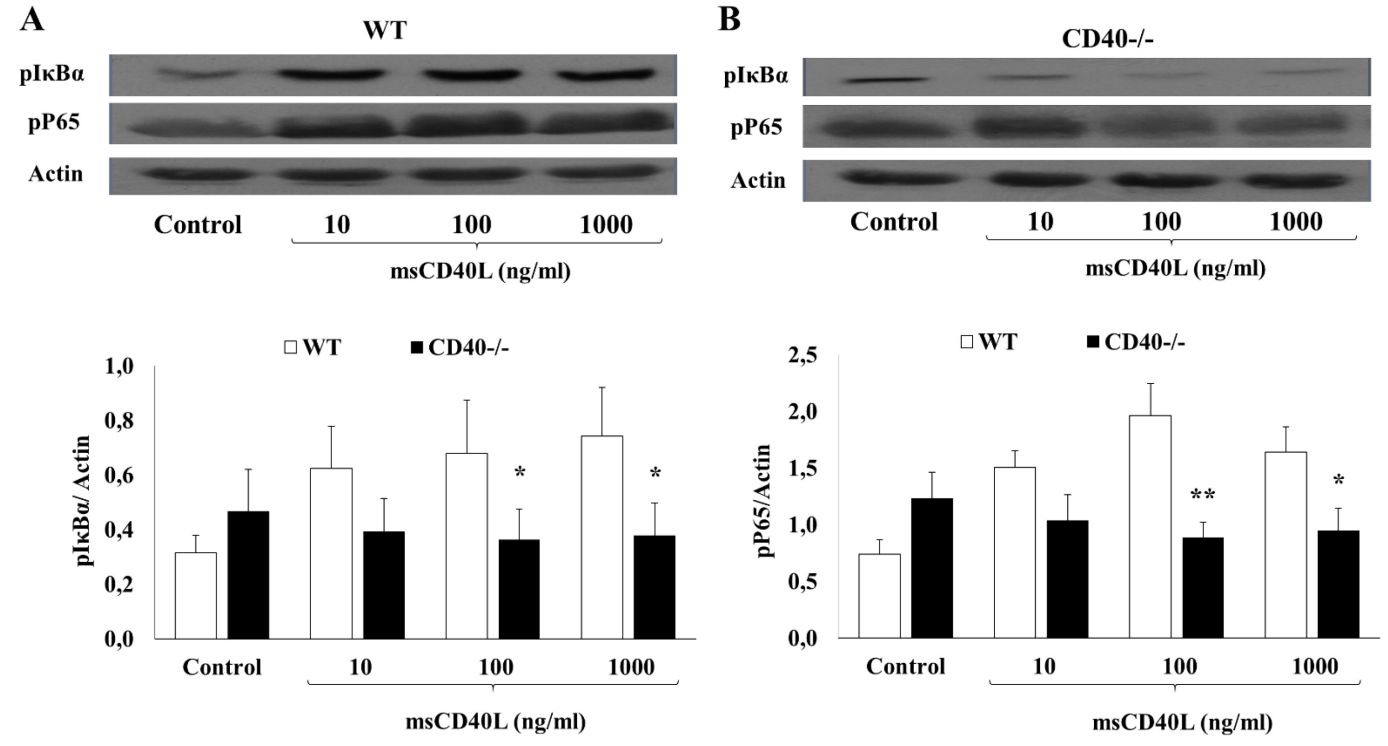
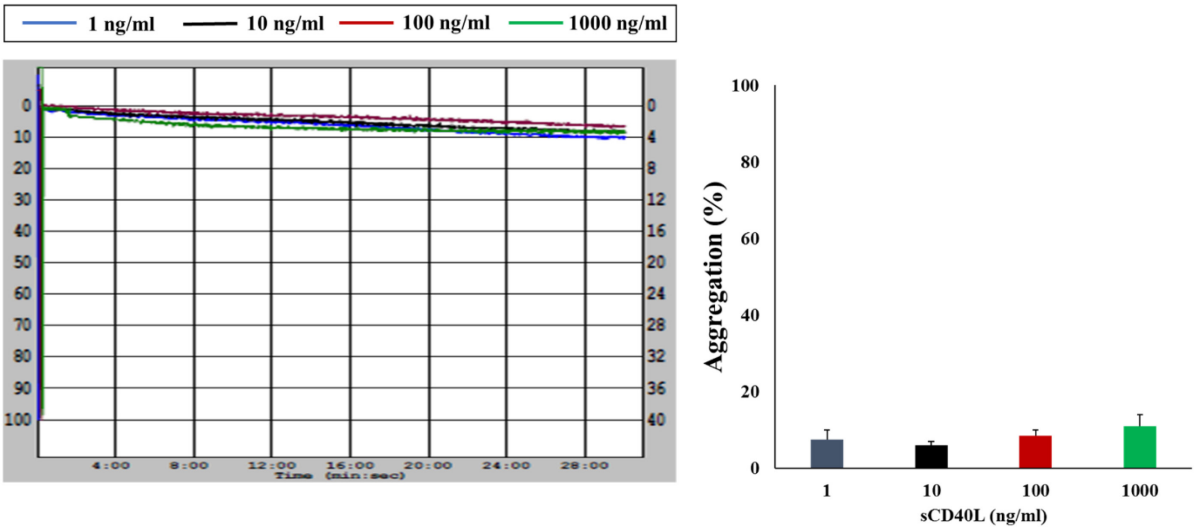


Figure 5

A


1 ng/ml
10 ng/ml
100 ng/ml
1000 ng/ml
Time (min:sec)
Aggregation (%)
sCD40L (ng/ml)

B

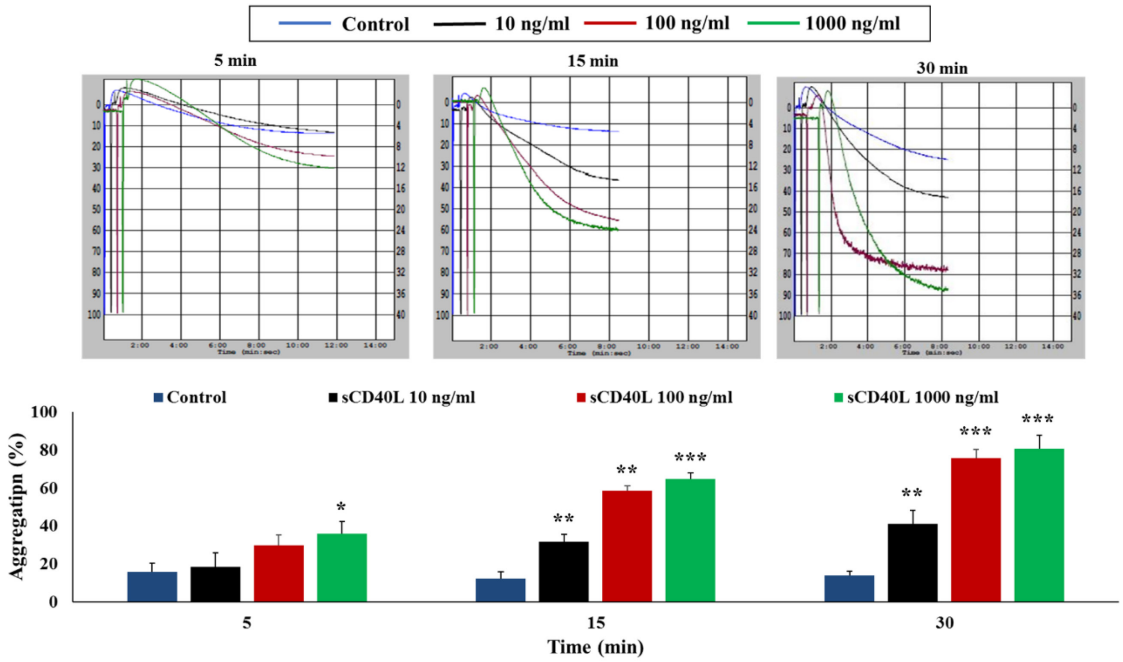
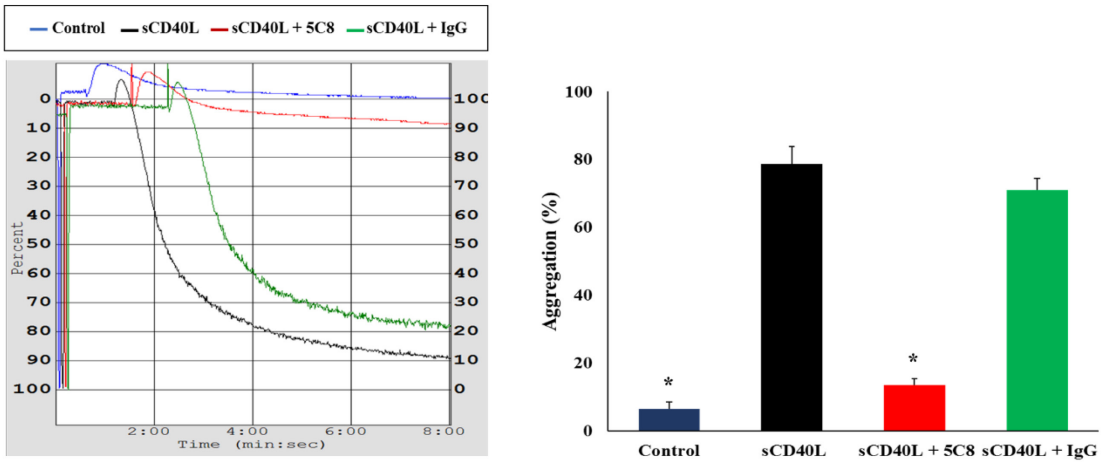
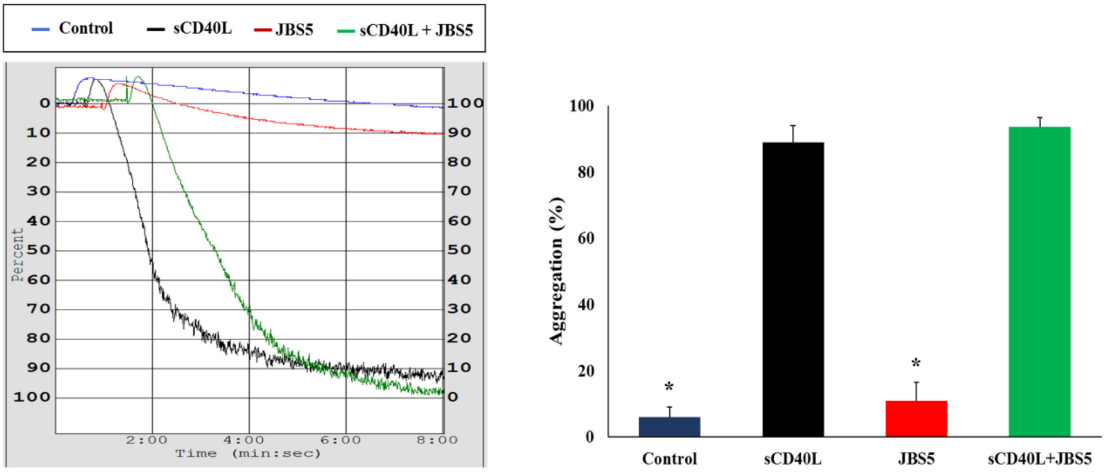

Control
10 ng/ml
100 ng/ml
1000 ng/ml
5 min
15 min
30 min
Control
sCD40L 10 ng/ml
sCD40L 100 ng/ml
sCD40L 1000 ng/ml
Aggregatipn (%)
Time (min)

Figure 6

A


Control
sCD40L
sCD40L + 5C8
sCD40L + IgG
Percent
Time (min:sec)
2:00
4:00
6:00
8:00
Aggregation (%)
Control
sCD40L
sCD40L + 5C8
sCD40L + IgG

B


Control
sCD40L
JBS5
sCD40L + JBS5
Percent
Time (min:sec)
2:00
4:00
6:00
8:00
Aggregation (%)
Control
sCD40L
JBS5
sCD40L+JBS5

C

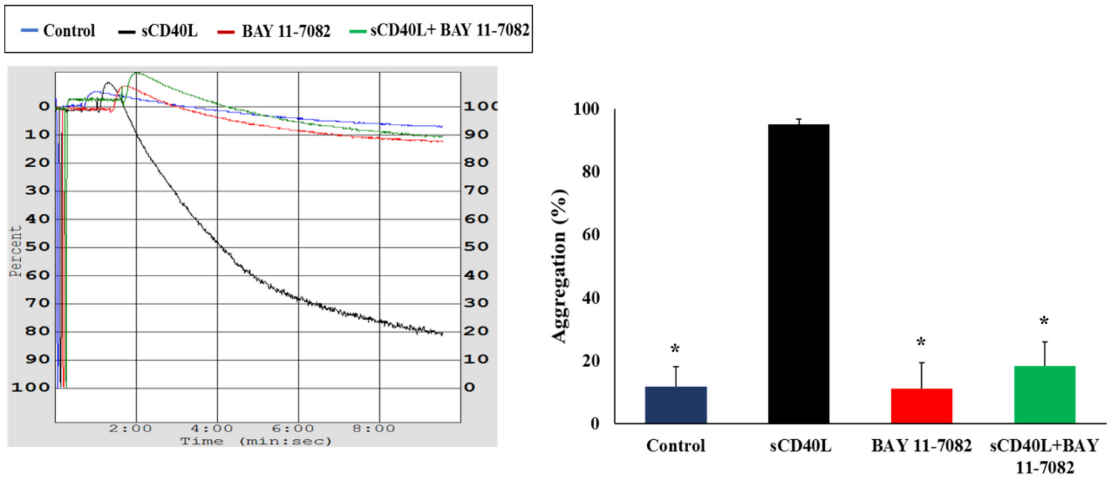

Control
sCD40L
BAY 11-7082
sCD40L+ BAY 11-7082
Percent
Time (min:sec)
2:00
4:00
6:00
8:00
Aggregation (%)
Control
sCD40L
BAY 11-7082
sCD40L+BAY 11-7082

Figure 7

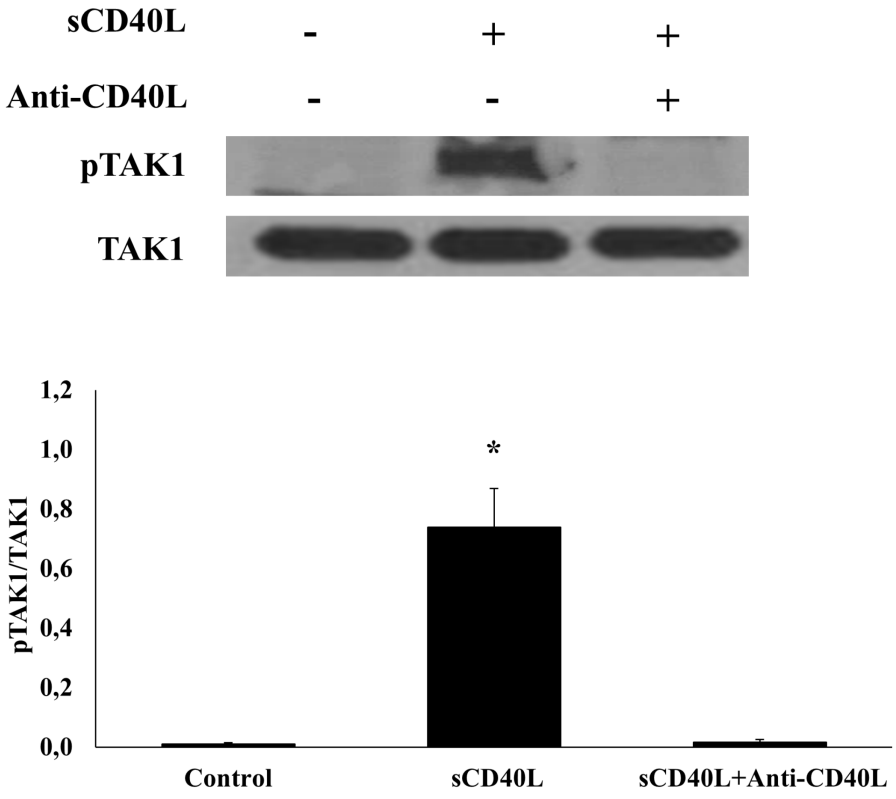

sCD40L
-
+
+
Anti-CD40L
-
-
+
pTAK1
TAK1
pTAK1/TAK1
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
Control
sCD40L
sCD40L+Anti-CD40L

Figure 8

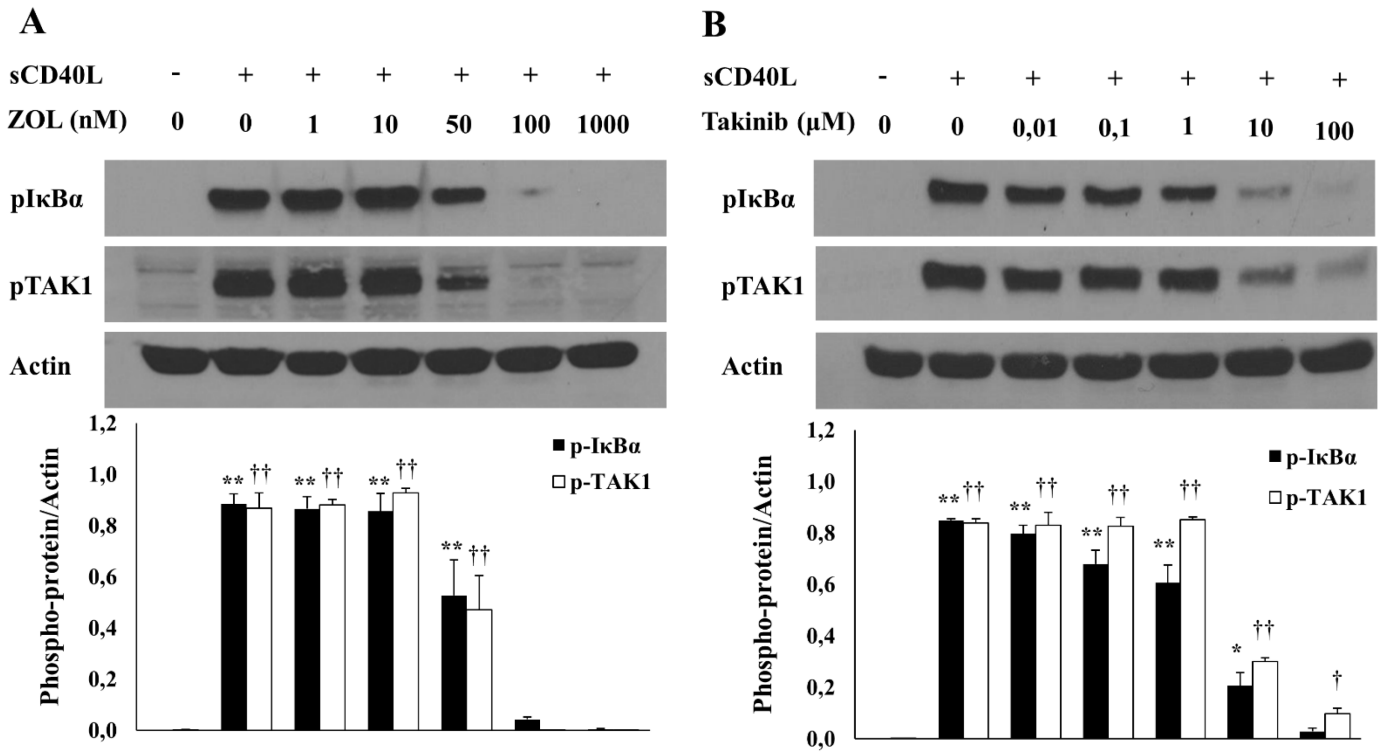
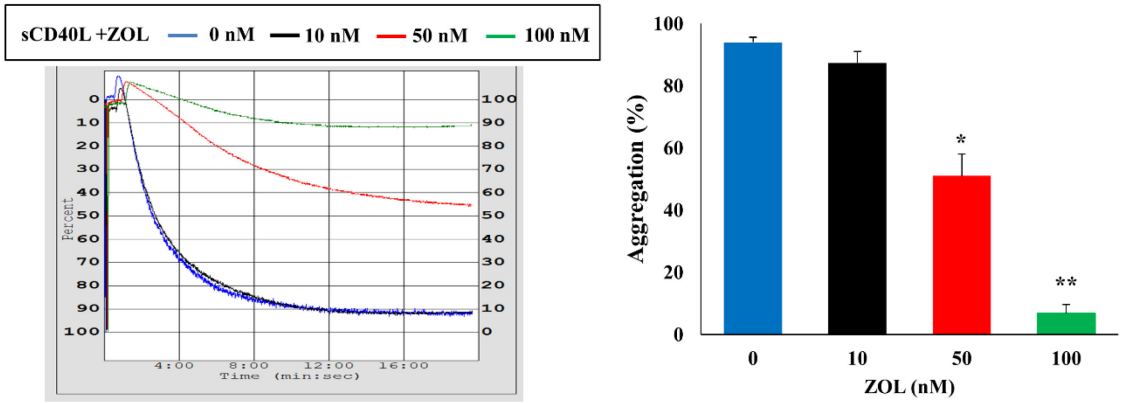
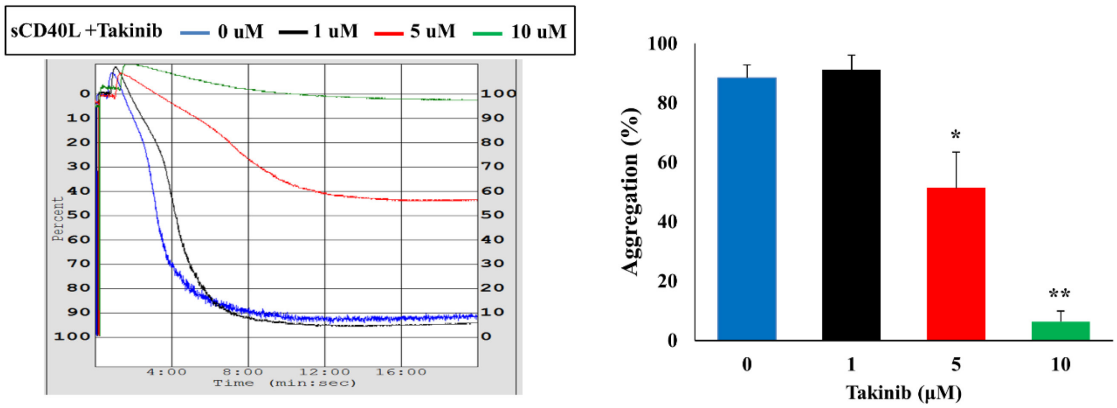

A
sCD40L - + + + + + +
ZOL (nM) 0 0 1 10 50 100 1000
pIκBα
pTAK1
Actin
Phospho-protein/Actin
1,2
1,0
0,8
0,6
0,4
0,2
0,0
p-IκBα
p-TAK1
B
sCD40L - + + + + + +
Takinib (μM) 0 0 0,01 0,1 1 10 100
pIκBα
pTAK1
Actin
Phospho-protein/Actin
p-IκBα
p-TAK1

Figure 9

A


sCD40L +ZOL
0 nM
10 nM
50 nM
100 nM
Percent
Time (min:sec)
4:00
8:00
12:00
16:00
Aggregation (%)
ZOL (nM)
0
10
50
100
*
**

B


sCD40L +Takinib
0 uM
1 uM
5 uM
10 uM
Percent
Time (min:sec)
4:00
8:00
12:00
16:00
Aggregation (%)
Takinib (μM)
0
1
5
10
*
**

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5.2 Deuxième Article: Publié dans Journal of American Heart Association en Janvier 2020

Aspirin reduces the potentiating effect of CD40L on platelet aggregation via inhibition of myosin light chain

Résumé

Mise en contexte: Le traitement antiplaquettaire à l'Aspirine (ASA) est moins efficace chez certains patients coronariens, ce qui augmente leur risque de développer une thrombose. Des taux sanguins élevés de médiateurs thrombo-inflammatoires, tels que le sCD40L, peuvent expliquer de telles variabilités. Nous avons émis l'hypothèse qu'en présence de taux élevés de sCD40L, l'efficacité de l'ASA peut varier sur la fonction plaquettaire. Ainsi, notre objectif principal était de déterminer les effets de l'ASA sur la signalisation de sCD40L et l'agrégation plaquettaire.

Méthodes et résultats: Les effets de l'ASA sur les plaquettes humaines traitées au sCD40L, en réponse à des concentrations sous-optimales de collagène ou de thrombine, ont été évalués sur l'agrégation, la sécrétion de thromboxane A_2 (TxA_2) et la phosphorylation de p38-MAPK, NF-κB, TGF- la kinase 1 activée par la protéine β (TAK-1) et la chaîne légère de la myosine (MLC). Le sCD40L a significativement augmenté la sécrétion de TxA_2 dans les plaquettes, en réponse à des doses sous-optimales de collagène et de thrombine. Cet effet a été inversé par l'ASA. L'ASA n'a pas inhibé la phosphoryltion de p38-MAPK, NF-κB, TAK-1, que ce soit avec une stimulation par sCD40L seul ou avec des agonistes plaquettaires. Le sCD40L a potentialisé l'agrégation plaquettaire, un effet complètement renversé et partiellement réduit par l'ASA en réponse à une dose sous-optimale de collagène et de thrombine, respectivement. Les effets de l'ASA sur les plaquettes traitées au sCD40L et stimulé avec du collagène étaient liés à l'inhibition du changement de forme des plaquettes et à la phosphorylation de la MLC.

Conclusion: L'ASA n'affecte pas la signalisation plaquettaire de sCD40L, mais empêche son effet sur la sécrétion de TxA_2 et l'agrégation plaquettaire en réponse au collagène, via un mécanisme impliquant l'inhibition de la MLC. Le ciblage de l'axe sCD40L dans les plaquettes

peut avoir un potentiel thérapeutique chez les patients présentant des taux élevés de CD40L et qui ne répondent pas ou moins à l'ASA.

Contribution des auteurs

Kevin Kojok: Planification et exécution des expériences d'ELISA, d'agrégation plaquettaire et de signalisation intracellulaire (Figures 1-4 et 6-8). Analyse des résultats et montage des figures. Rédaction et correction de l'article.

Mira Mohsen: Isolation du sang des donneurs, planification et exécution des expériences d'ELISA (Figure 1) et d'agrégation plaquettaire (Figure 5 et 7), ainsi que la rédaction et la correction de l'article.

Abed El Hakim El Kadiry: Participation aux techniques liées à la signalisation intracellulaire

Walid Mourad: Codirection générale

Yahye Merhi: Direction générale et correction de l'article.

Aspirin reduces the potentiating effect of CD40L on platelet aggregation via inhibition of myosin light chain

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Research Centre, Centre Hospitalier de l'Université de Montréal, Montréal, QC, Canada (W.M.).

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*: Both authors contributed equally to this work.

Running title: ASA in CD40L-primed platelets

Journal Subject Terms: Platelets, Cell Signaling/Signal Transduction, Thrombosis

Background—Antiplatelet therapy with Aspirin (ASA) is less efficient in some coronary patients, which increases their risk of developing thrombosis. Elevated blood levels of thrombo-inflammatory mediators, like sCD40L, may explain such variabilities. We hypothesized that in the presence of elevated levels of sCD40L, the efficacy of ASA may vary; and aimed to determine the effects of ASA on CD40L signaling and aggregation of platelets.

Methods and Results—The effects of ASA on CD40L-treated human platelets, in response to suboptimal concentrations of collagen or thrombin, were assessed on aggregation, thromboxane A2 (TxA_2) secretion, and phosphorylation of p38-MAPK, NF-κB, TGF-β-activated kinase 1 (TAK-1), and myosin light chain (MLC). sCD40L significantly elevated TxA_2 secretion in platelets, in response to suboptimal doses of collagen and thrombin, which was reversed by ASA. ASA did not inhibit phosphoryltion of p38-MAPK, NF-κB, TAK-1, either with sCD40L stimulation alone or with platelet agonists. sCD40L potentiated platelet aggregation, an effect completely reversed and partialy reduced by ASA in response to a suboptimal dose of collagen and thrombin, respectively. The effects of ASA in sCD40L-treated platelets with collagen were related to inhibition of platelet shape change and MLC phosphorylation.

Conclusion—ASA does not affect platelet sCD40L signaling, but prevents its effect on TXA_2 secretion and platelet aggregation in response to collagen, via a mechanism implying inhibition of MLC. Targeting sCD40L axis in platelets may have therapeutic potential in patients with elevated levels of sCD40L that are non- or less-responsive to ASA.

Key Words: Platelets • CD40L • Aspirin • Thromboxane A_2

Clinical Perspectives

What is new?

- ASA inhibits TxA_2 secretion of sCD40L-treated platelets stimulated by suboptimal doses of agonists.
- ASA has no effect on platelet sCD40L signaling.

- ASA inhibits the potentiating action of sCD40L on platelet aggregation, an effect related to inhibition of platelet shape change and MLC phosphorylation.

What are the clinical implications?

- Elevated levels of sCD40L in the blood of coronary patients may have an impact on ASA efficiency.
- Targeting sCD40L signaling might have a therapeutic potential within coronary patients that are non- or less-responsive to ASA.

Introduction:

Platelets play a key role in cardiovascular diseases; since they are at the center of atherothrombotic events by mediating thrombo-inflammatory responses that amplify atherosclerosis in the coronary arteries and its subsequent clinical complications, including ischemic heart disease, acute coronary syndrome (ACS), myocardial infarction and stroke [1-3]. Therefore, Antiplatelet agents have been a main focus of secondary prevention of cardiovascular diseases [4]. Aspirin (ASA) is the most widely used medicine for prevention of these diseases and reduces the risk of myocardial infarction, stroke and cardiovascular death in a broad range of patients at high risk for future cardiovascular events [5, 6]. Nevertheless, ASA only prevents approximately 25% of coronary events and ischemic strokes when used in secondary prevention [7]. ASA acts by irreversibly inhibiting the cyclooxygenase-1 (COX-1) enzyme that catalyzes the conversion of arachidonic acid to prostaglandins G1/G2, which are subsequently converted by thromboxane synthase to thromboxane A2 (TxA_2) [8]. A large number of patients continue to experience thrombo-embolic events despite ASA therapy in a phenomenon known as 'aspirin resistance', which could be due to several reasons such as drug interactions, pharmacokinetics of ASA [9] or polymorphisms affecting several proteins such as COX-1, TxA_2 synthesis and platelet receptors including αIIbβ3, α2β1, GPIb-IX-V [10-14].

Patients with inflammatory diseases exhibit higher than expected rates of ischemic cardiovascular events that are not attributable to common traditional risk factors, but rather to inflammatory stimuli that trigger pathways contributing to the pathogenesis of atherosclerosis.[15,16] Patients who are resistant to antiplatelet therapy have a hyperactive platelet

phenotype [17, 18], which may be related to elevated levels of circulating primers [19-21]. Platelet primers do not stimulate platelet activation, but, in combination with physiologic stimuli, significantly enhance platelet function [22]. One of these remarkable primers is the thrombo-inflammatory mediator CD40L and its classical receptor CD40 [23, 24], which have gained significant attention for their involvement in the pathogenesis of inflammation, atherosclerosis, and atherothrombosis [25-27].

CD40L, also known as CD154 or gp39, is a type II transmembrane protein and a member of the tumor necrosis factor (TNF) gene superfamily. It is expressed on a broad range of cells, mainly T cells and platelets [25]. Like other members of the TNF family, CD40L has a soluble form (sCD40L) that is mainly produced by platelets. This molecule was initially thought to be solely implicated in immune and inflammatory responses [28] as it was mostly expressed by immune cells [29]. However, CD40L has a much broader role. In particular, we have previously shown that enhanced levels of sCD40L exacerbate platelet activation and aggregation through the CD40/TRAF-2/Rac-1/p38 MAPK signaling pathways [30]. More recently, we have also shown that the priming action of sCD40L on platelets involves nuclear factor kappa B (NF-κB) [31], a central mediator of inflammation linked to thrombotic process [32], in a CD40- and TGF-β-activated kinase 1 (TAK1)-dependent manner [33].

Although CD40L has an important role as a platelet primer through its strong enhancement of platelet activation and aggregation in response to suboptimal concentrations of agonists, its effect may influence the anti-platelet efficiency of ASA. The objective of the present study was set to determine the effect of ASA on CD40L in platelet signaling, secretion and aggregation.

Methods

The data, analytic methods, and study materials will be made available, upon request from the corresponding author, to other researchers for the purpose of reproducing the results or replicating the procedure.

Preparation of human platelets

Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least three weeks before the experiment. All participants provided written informed consent, according to a protocol that was approved by the Human Ethical Committee of the Montreal Heart Institute, in accordance with the declaration of Helsinki for experiments involving humans. Washed platelets were prepared as previously described [30, 31]. Briefly, platelet-rich plasma (PRP) was obtained by centrifugation of acid citrate dextrose anticoagulated blood. Platelets were then pelleted from PRP, to which 1 ug/mL of PGE_1 was added, washed with HBSS-Hank's sodium citrate buffer, and finally re-suspended in HBSS-Hank's buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$. Platelets were rested at 37ºC for at least 30 minutes before further manipulation.

Measurement of Thromboxane A_2

The stable hydrolysis product of TxA_2, thromboxane B_2, was used as a readout of TxA_2 production. It was measured using a commercial Thromboxane B_2 ELISA kit (R&D systems). Platelet samples from the aggregation reactions were quenched at 5 min with 2 µM indomethacin and 5 mM EDTA to inhibit further production of TxA_2. Samples were centrifuged (5 min, 1000 rpm at 4ºC) and the supernatant was removed and stored at -80°C for subsequent analysis.

Phosphorylation of p38-MAPK, NF-κB, TAK-1, and MLC

Western blots were performed to assess the phosphorylation levels of p38-MAPK, NF-κB, TAK-1, and MLC. Briefly, platelets (1000 x 10^6/mL) were stimulated as indicated and lysed immediately by the addition of 1/4 volume of 4X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 5% β-mercaptoethanol. All samples were boiled for 5 min. Protein lysates were then resolved in 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk for 1 hour, washed three times with TBS/T (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1 % Tween-20) and incubated with appropriate primary antibody overnight at 4ºC. We used primary antibodies against phospho-p38-MAPK $^{\text{threonine } 180/182}$, phospho-IκBα $^{\text{serine } 32/36}$, phospho-TAK1 $^{\text{threonine } 184/187}$, phospho-MLC $^{\text{serine } 19 \text{ threonine } 18}$, and β-actin (Cell Signaling Technology).

Following washing steps, membranes were labelled with horseradish peroxidase-conjugated secondary antibody for 1 hour, washed and bound peroxidase activity was detected by enhanced chemiluminescence (PerkinElmer Life Sciences). All membranes were stripped and reprobed for β-actin, a commonly chosen loading control. Data were presented as ratios of phosphorylated proteins to respective β-actin.

Measurement of platelet aggregation

We monitored aggregation of washed human platelets on a four-channel optical aggregometer (Chronolog Corp.) under shear (1000 rpm) at 37ºC. Platelet suspensions (250 x 10^6/mL) were pretreated with ASA (30 uM, Tocris)[8, 34] or ML7 (Selective inhibitor of myosin light chain kinase, 50 uM, Tocris)[35] for 5 minutes followed by treatment with sCD40L (1000 ng/mL, R&D systems) for 30 minutes at 37ºC [33] . After that, platelet aggregation was triggered by a suboptimal dose of collagen (0.25 ± 0.1 µg/ml, Chronolog Corp.), or α-thrombin (0.025 ± 0.01 U/mL. Sigma-Aldrich). The suboptimal dose of agonist that does not induce more than 30% aggregation was selected before each experiment from a dose-response curve of platelet aggregation in response to collagen or thrombin (Supplemental Figure 1). Traces were recorded until stabilization of platelet aggregation [30, 31, 36].

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 25. Results are presented as median ± IQR (Interquartile Range). Statistical comparisons were done using the Kruskal-Wallis test followed by Dunn's post hoc test. . The specific statistical tests used, the median of data, the number (n) of experiments, and the P values are specified in the figure legends. A p-value < 0.05 was considered statistically significant.

Results

ASA inhibits thromboxane A_2 secretion induced by sCD40L

We investigated whether sCD40L increases TxA_2 formation and whether ASA might reverse this effect. In unstimulated platelets, the production of TxA_2 (4.5 ± 3.1 ng/ml) was unaffected by sCD40L in the presence or absence of ASA (Fig. 1A). In stimulated platelets, the production of TxA_2 increased to 15.7 ± 6.3 ng/ml in response to a suboptimal concentration of collagen (Fig. 1C), which was reduced significantly by 5 folds to 3.5 ± 3.1 in the presence of ASA. Likewise, TxA_2 levels remain unchanged (5.9 ± 3.9 ng/ml) with ASA pretreatment in response to a suboptimal concentration of thrombin (Fig. 1B). However, sCD40L significantly elevated TxA_2 production in platelets in the presence of suboptimal doses of collagen (23.2 ± 8,1 ng/ml, Fig. 1C) and thrombin (24.2 ± 7.9 ng/ml, Fig. 1B), an effect that was completely reversed by ASA.

ASA does not affect CD40L signaling

We have previously shown that NF-κB, p38-MAPK, and TAK1 are required for sCD40L-induced platelet activation and potentiation of platelet aggregation [30, 31, 33]. We aimed to verify whether the impact of ASA passes via modulation of sCD40L signaling in platelets. To this end, we assessed the phosphorylation levels of IκBα (Fig. 2), p38-MAPK (Fig. 3), and TAK1 (Fig. 4). We have first showed that sCD40L induced robust phosphorylations of IκBα, p38-MAPK and TAK1, which were not affected by the addition of suboptimal concentrations of collagen or thrombin. Treatment of platelets with ASA has no impact on the phosphorylation of those proteins, either following sCD40L stimulation alone or with sCD40L and a suboptimal dose of collagen or thrombin.

ASA reduces the effect of CD40L on platelet aggregation

sCD40L alone was unable to induce platelet aggregation (Fig. 5A), but potentiated platelet aggregation in response to suboptimal doses of collagen (Fig. 5B) or thrombin (Fig. 5C). The effect of sCD40L in potentiating platelet aggregation was completely reversed by ASA in response to a suboptimal dose of collagen. However, the potentiating impact of sCD40L on

platelet aggregation was significantly reduced by approximately 50% by ASA when sCD40L pretreated-platelets were stimulated with a suboptimal dose of thrombin. Interestingly, only the effect of ASA in response to collagen and sCD40L was associated with complete inhibition of platelet shape change (Fig. 5B, red and green aggregation traces) that precedes the onset of the aggregation process.

Involvement of MLC in ASA action

To get insight on the observed effect of ASA on platelet shape changes in response to collagen, we assessed the phosphorylation of Myosin light chain (MLC), a regulator of the dynamic remodeling of the actin cytoskeleton required for platelet shape change [37-39]. As shown in Figure 6, ASA inhibits MLC phosphorylation induced by a suboptimal aggregating dose of collagen in sCD40L-treated platelets. However, in sCD40L-treated platelet stimulated with a suboptimal dose of thrombin, ASA was without any significant effect. To understand the impact of such findings on platelet function, we used ML7, a selective inhibitor of MLC kinase, in platelet aggregation tests. A concentration of 50 uM of ML7 was able to abolish MLC activation in platelets (Supplemental Figure 2). ML7 inhibited platelet aggregation and shape change when platelets are stimulated with suboptimal dose of collagen or thombin and thereby prevented the potentiating action of sCD40L on platelet aggregation (Fig. 7). Finally, similar to ASA, ML7 has no effect of the phosphorylation of IκBα, p38-MAPK and TAK1 (Fig. 8).

Discussion

In this study, we hypothesized that in the presence of elevated levels of sCD40L, like those found in the circulation of coronary patients, reducing the influence of sCD40L on platelet activity should improve antiplatelet efficacy. To address our hypothesis, we assessed the effects of ASA on sCD40L-induced signalling, secretion and aggregation of unstimulated and primed platelets with suboptimal concentrations of physiological agonists, thrombin and collagen. We found that ASA is highly effective in completely reversing the potentiating effect of sCD40L on platelet aggregation in response to collagen. However, the response of sCD40L in response to thrombin was partially reduced. Such effect was independent of sCD40L signaling via NF-

κB, p38-MAPK or TAK1, but related to inhibition of MLC phosphorylation and platelet shape change in response to collagen of sCD40L-primed platelets.

The priming role of sCD40L, which potentiated significantly the aggregation in accordance with our previous studies [30, 31, 33], was reversed completely by pre-treatment with ASA, when platelets are challenged by collagen. However, sCD40L was able to overcome partially but significantly the inhibitory effect of ASA when aggregation was induced with suboptimal doses of thrombin. This could be explained by the ability of ASA to inhibit the effects of collagen on platelets, which relies principally on TxA_2 [40]. Whereas, thrombin is known to be a potent platelet activator [41, 42], independently of TxA_2. Indeed, despite dual therapies with antagonists of ADP receptors and ASA, a large number of ACS patients experience recurrent major cardiovascular ischemic events due to markers of thrombin activation remaining high after an ACS [43, 44]. This suggests a maintained platelet activation through a thrombin-mediated mechanism [45]. Interestingly, the effect of ASA was related to inhibition of collagen-induced platelet shape changes, which frequently occur even when platelets are exposed to minimal degrees of activation that cannot result in aggregation or adhesion, such as suboptimal concentrations of platelet agonists or shear stress [46]. The effect of ASA on platelet shape changes correlated with its effect on MLC phosphorylation, a major key player protein in platelet shape changes [37-39], that was also inhibited in response to a suboptimal concentration of collagen, but not thrombin; and those whether the platelet was stimulated with CD40L or not. This indicates that ASA acts exclusively on collagen through inhibition of the MLC. Such statement was confirmed with ML7, a selective inhibitor of MLC kinase, where we observed that MLC inhibition does not interfere in sCD40L signaling pathway. Furthermore, ML7 was able to inhibit sCD40L potentiating action in response to suboptimal doses of collagen and thrombin. In fact, MLC is major player in platelet activation and aggregation [47, 48]. However, ASA has no effect on MLC activation in thrombin-stimulated platelets, which translates to a maintained shape change and sCD40L-potentiation action in the aggregation process. This could be explained by the ability of thrombin to induce a shape change in a TxA_2 independent manner. By measuring TxB_2 secretion, we observed that sCD40L is capable of enhancing TxA_2 secretion in response to suboptimal doses of agonists. Thus, CD40L, aside from being able to enhance platelet alpha-granule release [24], can also amplify TxA_2 secretion. This observation is consistent with the results of a previous study where

other platelet primers also enhanced TxA_2 secretion [34]. Interestingly, following inhibition with ASA, sCD40L maintains its ability to trigger platelet aggregation in response to suboptimal doses of thrombin indicating that the priming action of sCD40L is independent of TxA_2. In fact, one of the proposed mechanisms by which ASA resistance develops is via activation of alternative signaling pathways that are TxA_2-independent [49]. Also, our results demonstrate that this resistance may be explained by the fact that ASA does not affect sCD40L signaling. Indeed, ASA seems to have an indirect effect in preventing sCD40L potentiation action in platelet aggregation by inhibiting collagen action that is dependent on TxA_2 secretion which is not the case with thrombin that can induce aggregation independently of TxA_2 [50]. Furthermore, it has been shown that ASA inhibits sCD40L release in collagen-stimulated platelets of patients undergoing ASA treatments, but does not affect the release of sCD40L when platelets of those patients are stimulated with a PAR agonist that has a similar effect to thrombin[51, 52]. Our results with TxA_2 production corroborate those previous findings obtained with sCD40L, as ASA inhibits the release of TxA_2 in collagen-, but not thrombin-, stimulated platelets. This may also explain why the potentiation of aggregation in sCD40L primed platelets was inhibited after collagen-, but not thrombin- stimulation. Our previous work showed that sCD40L signaling in platelets goes through two pathways: the CD40/TAK-1/NF-κB pathway [31, 33] and the Rac1/p38 MAPK [30]. These pathways remained activated when ASA treated platelets are stimulated with sCD40L indicating that sCD40L-mediated resistance to ASA is dependent on TAK-1, NF-κB, and p38 MAPK.

To translate our findings to a clinical setting, our results suggest that patients undergoing anti-platelet therapy that blocks TxA2 formation are not totally protected from the priming impact of sCD40L on platelets, suggesting that elevated sCD40L may reduce the efficacy of antiplatelet therapies. Thus, revealing the priming mechanisms of the CD40L/CD40 axis in platelet function may help identifying molecular targets in its signaling pathways that may emerge as innovative preventive treatments in coronary patients with elevated circulating levels of sCD40L. The major clinical outcome is to improve the management of atherothrombotic events in CAD patients that are none or less responding to conventional antiplatelet therapies. This will add significant value to the healthcare system beyond current prevention programs and existing traditional therapies in CAD. Indeed, perspective studies may be planned in patients with

metabolic syndrome at high risk of CAD and knowing to have high levels of circulating sCD40L. In addition, it would be also benefice to surmise finding a subgroup of patients that are none or less responding to antiplatelet therapies and whose platelets can be manipulated in vitro with an experimental treatment that interferes with sCD40L/CD40 axis. The receptor CD40, its adaptor protein TRAF2, the downstream effectors TAK1/NF-κB, and Rac/p38 MAPK have been involved in sCD40L/CD40 axis. However the specific pathways that can be targeted pharmacologically in platelets, without affecting other physiological functions, still to be identified and characterized in further studies.

In conclusion, ASA does not affect platelet sCD40L signaling, but prevents its effect on TXA2 secretion and platelet aggregation in response to collagen, via a mechanism implying inhibition of platelet shape change and MLC phosphorylation. Elevated levels of sCD40L in the blood of coronary patients may have an impact on ASA efficiency. Targeting sCD40L axis in platelets may have therapeutic potential in patients with elevated levels of CD40L that are none- or less-responsive to ASA.

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Disclosures

None.

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Figure Legends

Figure 1. ASA inhibits sCD40L-induced Thromboxane B_2 formation in platelets in response to a suboptimal dose of agonists. Washed human platelets (250 x 10^6/mL) were left untreated or pretreated with ASA (30 μM) for 5 minutes at 37°C. They were then stimulated with sCD40L (1000 ng/ml) or not for 30 minutes. Platelets were left unstimulated (A) or challenged with a suboptimal dose of thrombin (0,025 U/mL ± 0,01) (B) or collagen (0,25 ug/mL ± 0,1) (C). Aggregation was stopped with indomethacin (2 uM), 5 min following the adding of suboptimal doses of agonists. Platelets were centrifuged at 10000 rpm for 5 min at 4ºC and supernatant was collected. Thromboxane B_2 in the supernatant was then measured using a Thromboxane B_2 ELISA kit. (n = 10, median ± IQR). *p<0,05 *vs.* other treatements (Kruskal-Wallis followed by Dunn's post hoc test).

Figure 2. ASA does not affect NF-κB activation by sCD40L. Washed human platelets (1,000 x 10^6/mL) were pretreated or not with ASA (30 uM) for 10 min then stimulated with sCD40L (1000 ng/mL) and thrombin (0,025 U/mL) or collagen (0,25 ug/mL) for 5 minutes. Platelet lysates were resolved in 10% SDS–PAGE and assessed for p-IκBα. Actin blot is from stripped membranes of p-IκBα blot. Histograms represent the median of blot data, expressed in optical density (n = 4, median ± IQR). *p<0.01 *vs.* Control (Kruskal-Wallis followed by Dunn's post hoc test).

Figure 3. ASA does not affect p38-MAPK activation by sCD40L. Washed human platelets (1,000 x 10^6/mL) were pretreated or not with ASA (30 uM) for 10 min then stimulated with

sCD40L (1000 ng/mL) and thrombin (0,025 U/mL) or collagen (0,25 ug/mL) for 5 minutes. Platelet lysates were resolved in 10% SDS–PAGE and assessed for p-p38-MAPK. Actin blot is from stripped membranes of p-p38-MAPK blot. Histograms represent the median of blot data, expressed in optical density (n = 4, median ± IQR). *p<0.01 *vs.* Control (Kruskal-Wallis followed by Dunn's post hoc test).

Figure 4. ASA does not affect TAK1 activation by sCD40L. Washed human platelets (1,000 x 10^6/mL) were pretreated or not with ASA (30 uM) for 10 min then stimulated with sCD40L (1000 ng/mL) and thrombin (0,025 U/mL) or collagen (0,25 ug/mL) for 5 minutes. Platelet lysates were resolved in 10% SDS–PAGE and assessed for p-TAK1. Actin blot is from stripped membranes of p-TAK1 blot. Histograms represent the median of blot data, expressed in optical density (n = 4, median ± IQR). *p<0.01 *vs.* Control (Kruskal-Wallis followed by Dunn's post hoc test).

Figure 5. Effects of **s**CD40L and ASA on platelet aggregation in response to suboptimal doses of agonists. Washed human platelets (250 x 10^6 /mL) were left untreated or pretreated with ASA (30 μM) for 5 minutes at 37°C. In A, sCD40L (1000 ng/ml) was used as the platelet agonist, whereas in B and C, platelets were preincubated with sCD40L or not for 30 minutes. Platelet aggregation was induced with suboptimal doses of collagen (0.25 ug/ml,B) or thrombin (0.025 U/ml,C). Histograms represent medians of aggregation percentages (n = 25, median ± IQR). *p<0.01 *vs.* other treatments (Kruskal-Wallis followed by Dunn's post hoc test).

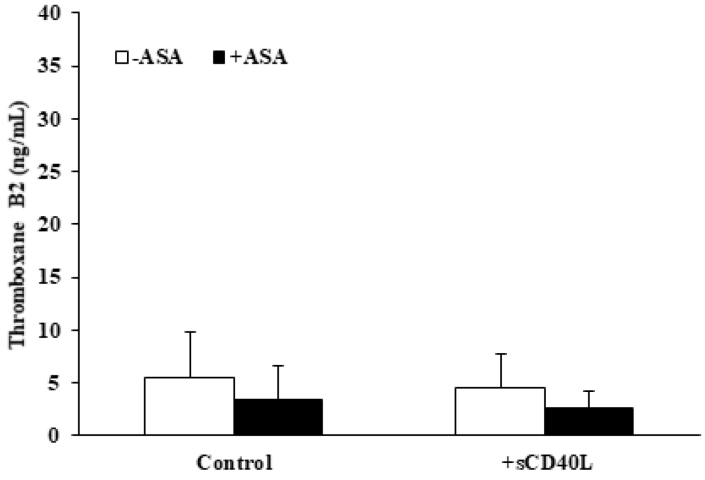
Figure 6. ASA inhibits MLC phosphorylation in platelets stimulated with collagen but not thrombin. Washed human platelets (1,000 x 10^6/mL) were pretreated or not with ASA (30 uM) for 10 minutes then stimulated with sCD40L (1000 ng/mL). Aggregation was triggered with suboptimal doses of thrombin (0,025 U/mL) or collagen (0,25 ug/mL). Platelet lysates were resolved in 15% SDS–PAGE and assessed for p-MLC. MLC blot is from stripped membranes of p-MLC blot. Histograms represent the median of blot data, expressed in optical density (n = 4, median ± IQR). *p<0.01 *vs.* Control (Kruskal-Wallis followed by Dunn's post hoc test).

Figure 7. Effect of ML7 on the potentiating action of sCD40L on platelet aggregation. Washed human platelets (250 x 10^6 platelets/ml) were left untreated or pretreated with ML7 (50 μM) for 5 minutes at 37°C. They were preincubated with sCD40L or not (1000 ng/ml) for 30 minutes. Platelet aggregation was induced with suboptimal doses of collagen (0.25 ug/ml, A) or thrombin (0.025 U/ml, B) . Histograms represent medians of aggregations (n = 9, median ± IQR). *p<0.01 *vs.* other treatments (Kruskal-Wallis followed by Dunn's post hoc test).

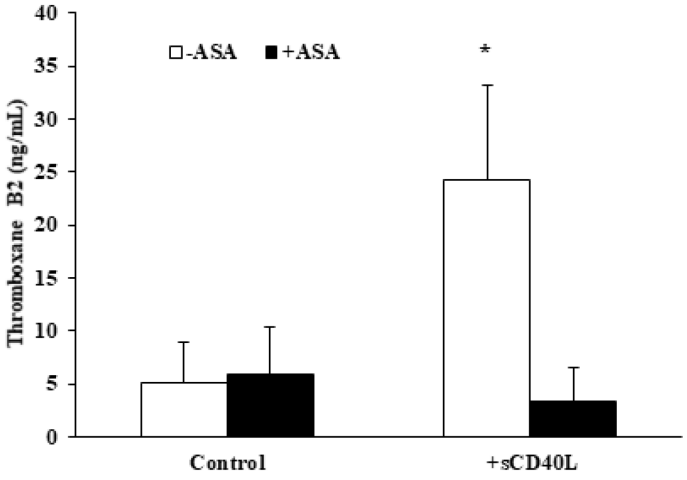
Figure 8. ML7 has no effect on sCD40L signaling. Washed human platelets (1,000 x 10^6/mL) were pretreated or not with ML7 (50 uM) for 5 min, then left untreated or stimulated with sCD40L (1000 ng/mL) for 5 minutes. Platelet lysates were resolved in 10% SDS–PAGE and assessed for p-TAK1 and stripped for p-IκBα, p-p38, and actin. Histograms represent the median of blot data, expressed in optical density (n = 5, median ± IQR). *p<0.01 *vs.* Control (Kruskal-Wallis followed by Dunn's post hoc test).

Figure 1

A


□-ASA ■+ASA
Thromboxane B2 (ng/mL)
40
35
30
25
20
15
10
5
0
Control
+sCD40L

B


□-ASA ■+ASA
*
Thromboxane B2 (ng/mL)
40
35
30
25
20
15
10
5
0
Control
+sCD40L

C

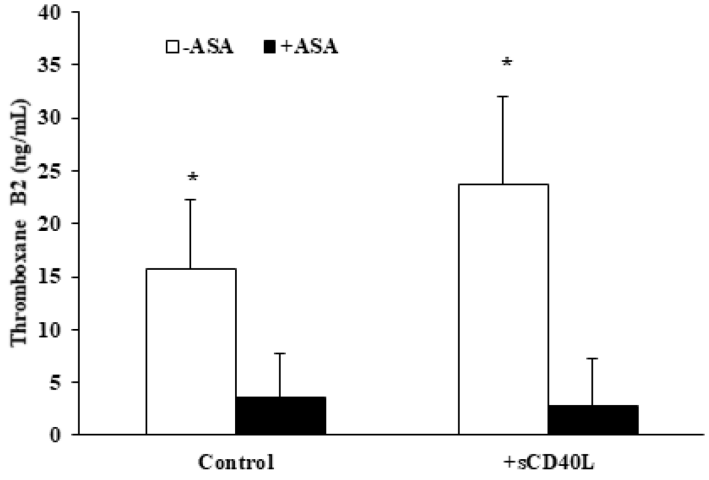

□-ASA ■+ASA
*
*
Thromboxane B2 (ng/mL)
40
35
30
25
20
15
10
5
0
Control
+sCD40L

Figure 2

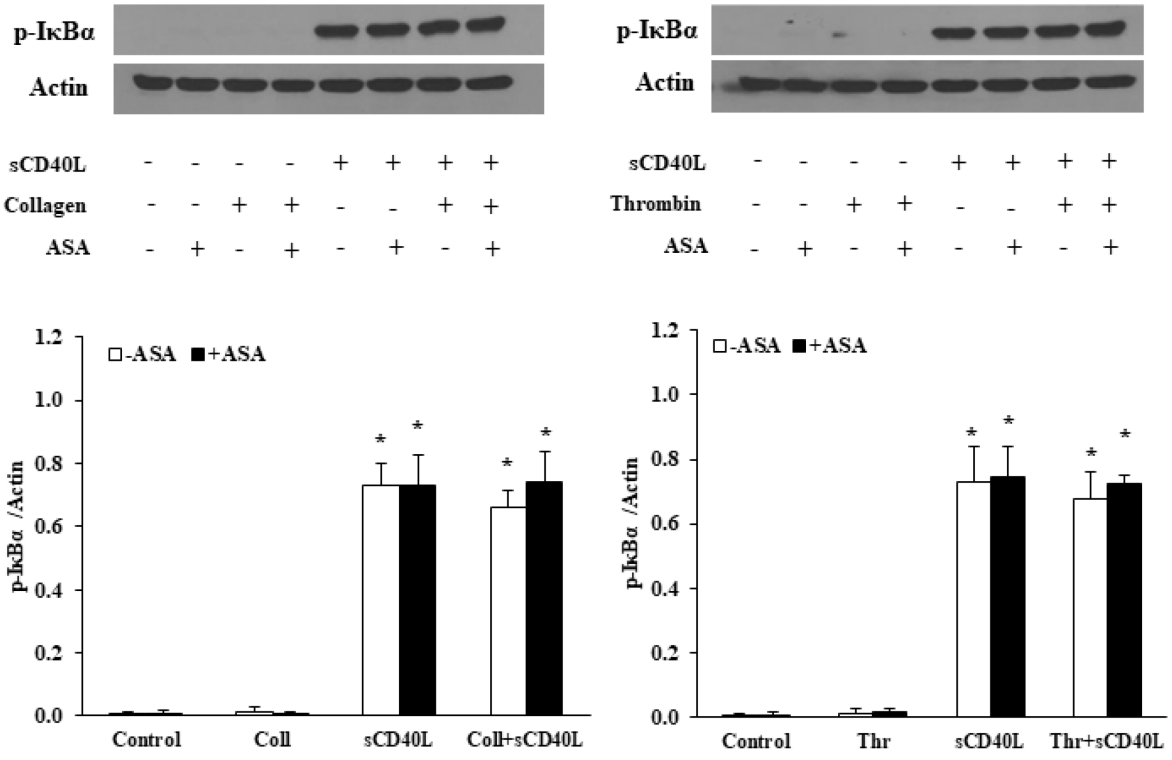
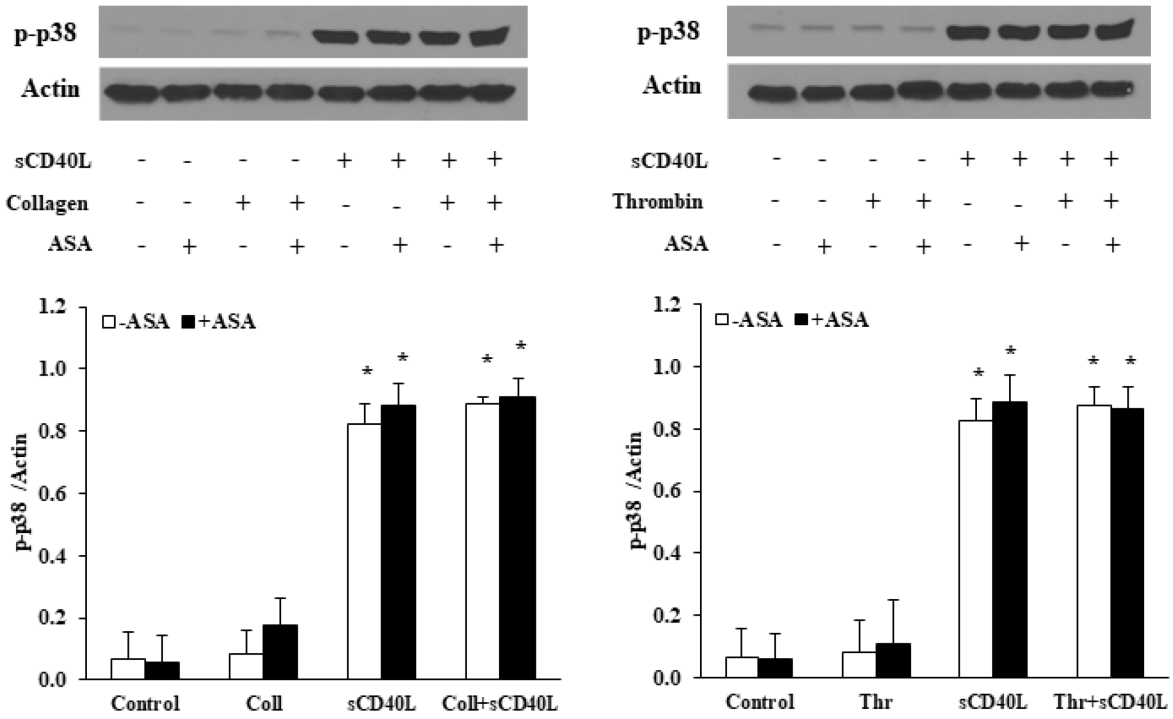

p-IκBα
Actin
sCD40L - - - - + + + +
Collagen - - + + - - + +
ASA - + - + - + - +
p-IκBα
Actin
sCD40L - - - - + + + +
Thrombin - - + + - - + +
ASA - + - + - + - +
□-ASA ■+ASA
p-IκBα /Actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Coll
sCD40L
Coll+sCD40L
□-ASA ■+ASA
p-IκBα /Actin
Control
Thr
sCD40L
Thr+sCD40L

Figure 3


p-p38
Actin
sCD40L - - - - + + + +
Collagen - - + + - - + +
ASA - + - + - + - +
-ASA +ASA
p-p38 /Actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Coll
sCD40L
Coll+sCD40L
p-p38
Actin
sCD40L - - - - + + + +
Thrombin - - + + - - + +
ASA - + - + - + - +
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Thr
sCD40L
Thr+sCD40L

Figure 4

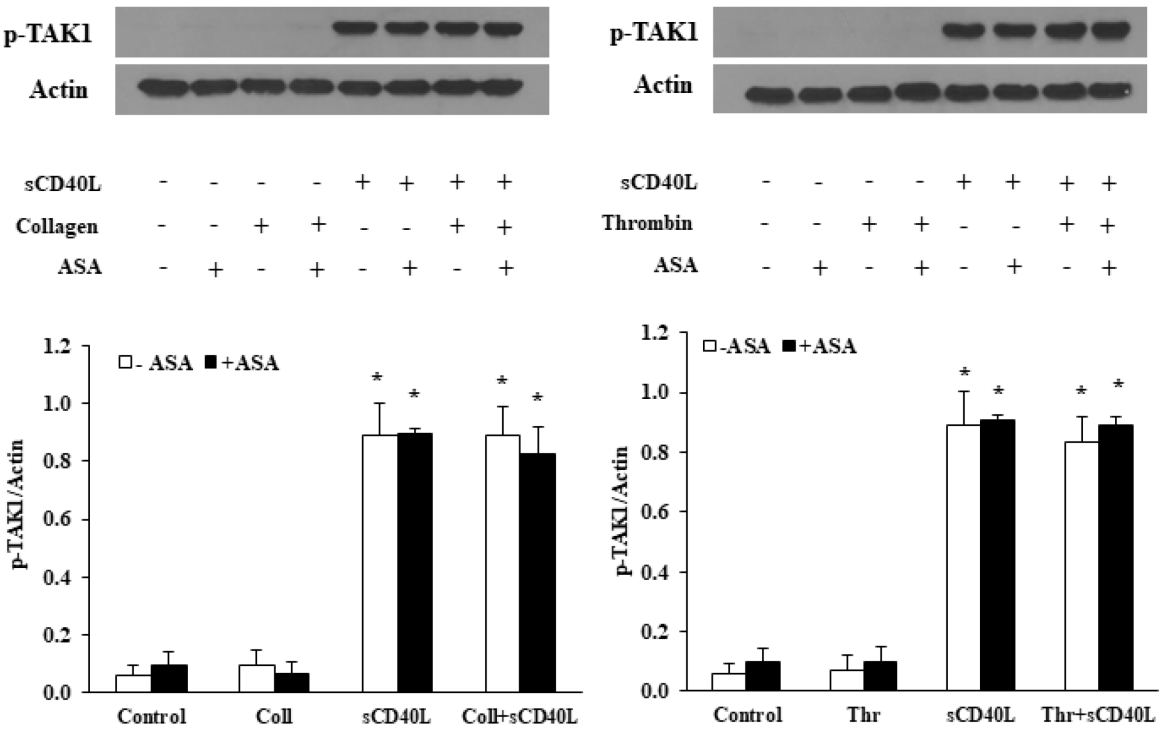

p-TAK1
Actin
sCD40L - - - - + + + +
Collagen - - + + - - + +
ASA - + - + - + - +
p-TAK1/Actin
□- ASA ■+ASA
Control
Coll
sCD40L
Coll+sCD40L
p-TAK1
Actin
sCD40L - - - - + + + +
Thrombin - - + + - - + +
ASA - + - + - + - +
p-TAK1/Actin
□-ASA ■+ASA
Control
Thr
sCD40L
Thr+sCD40L

Figure 5

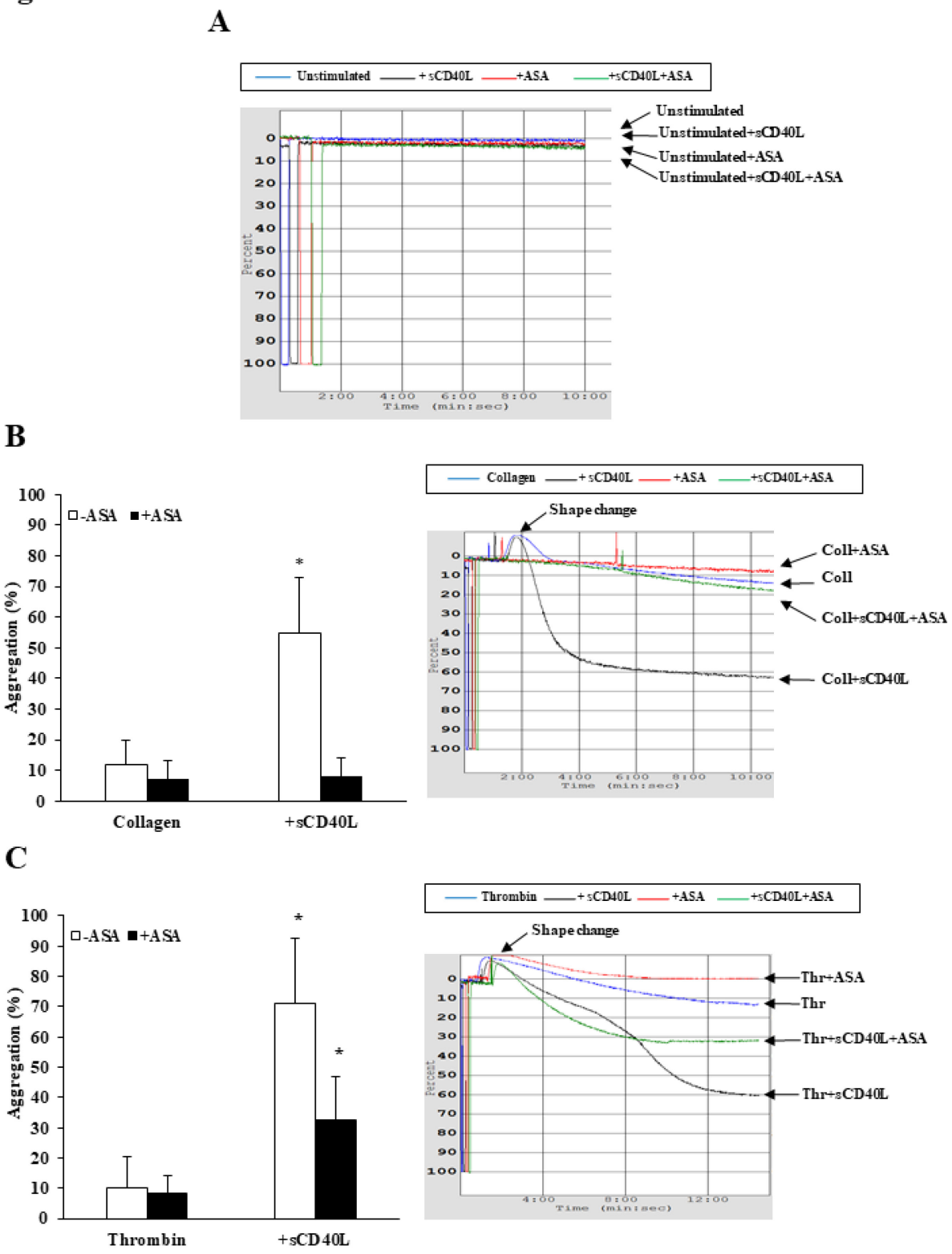

A
Unstimulated
+sCD40L
+ASA
+sCD40L+ASA
Unstimulated
Unstimulated+sCD40L
Unstimulated+ASA
Unstimulated+sCD40L+ASA
Percent
Time (min:sec)
B
-ASA
+ASA
Aggregation (%)
Collagen
+sCD40L
Collagen
Shape change
Coll+ASA
Coll
Coll+sCD40L+ASA
Coll+sCD40L
C
Thrombin
Thr+ASA
Thr
Thr+sCD40L+ASA
Thr+sCD40L

Figure 6

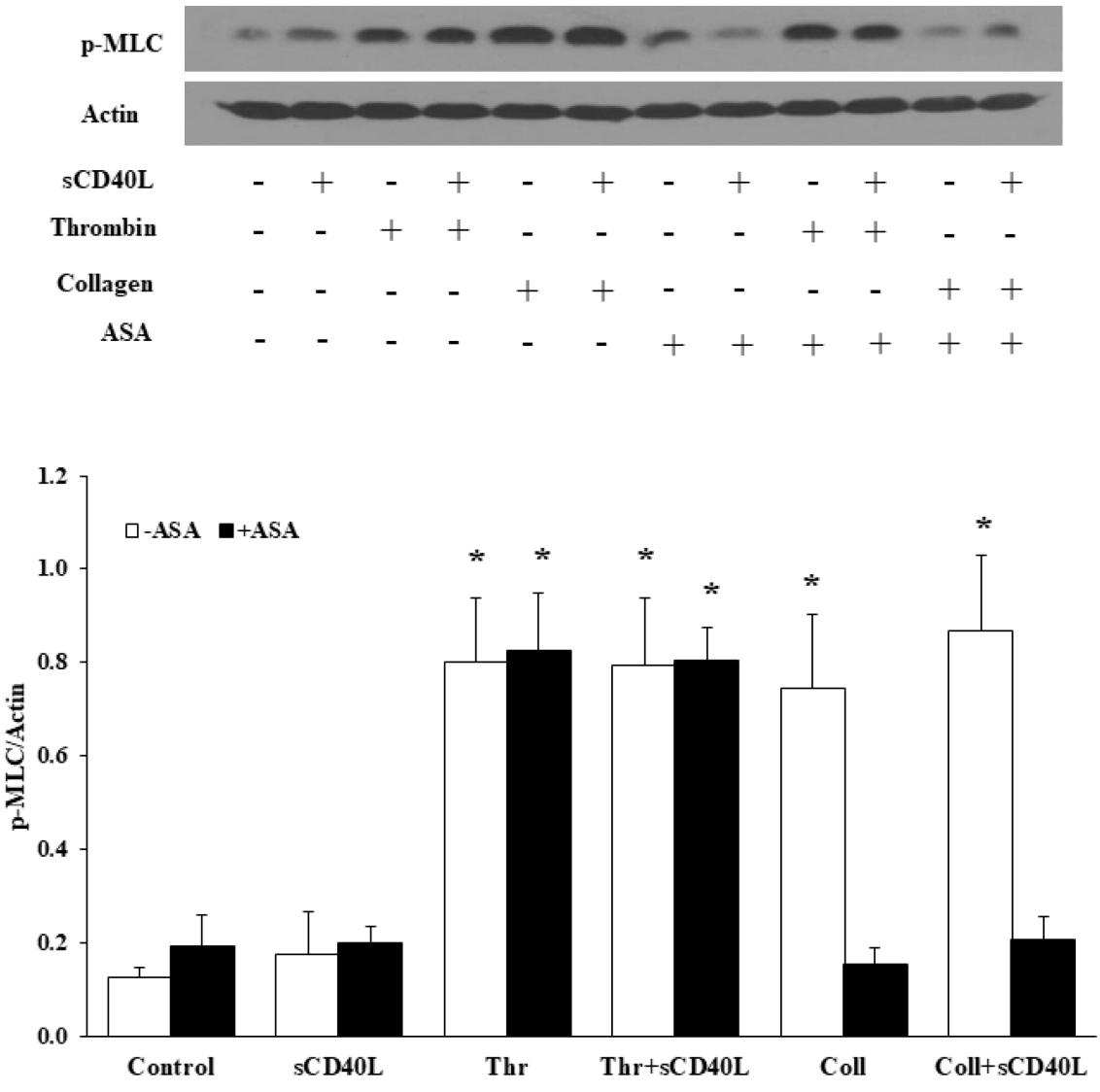

p-MLC
Actin
sCD40L - + - + - + - + - + - +
Thrombin - - + + - - - - + + - -
Collagen - - - - + + - - - - + +
ASA - - - - - - + + + + + +
-ASA +ASA
p-MLC/Actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
sCD40L
Thr
Thr+sCD40L
Coll
Coll+sCD40L

Figure 7

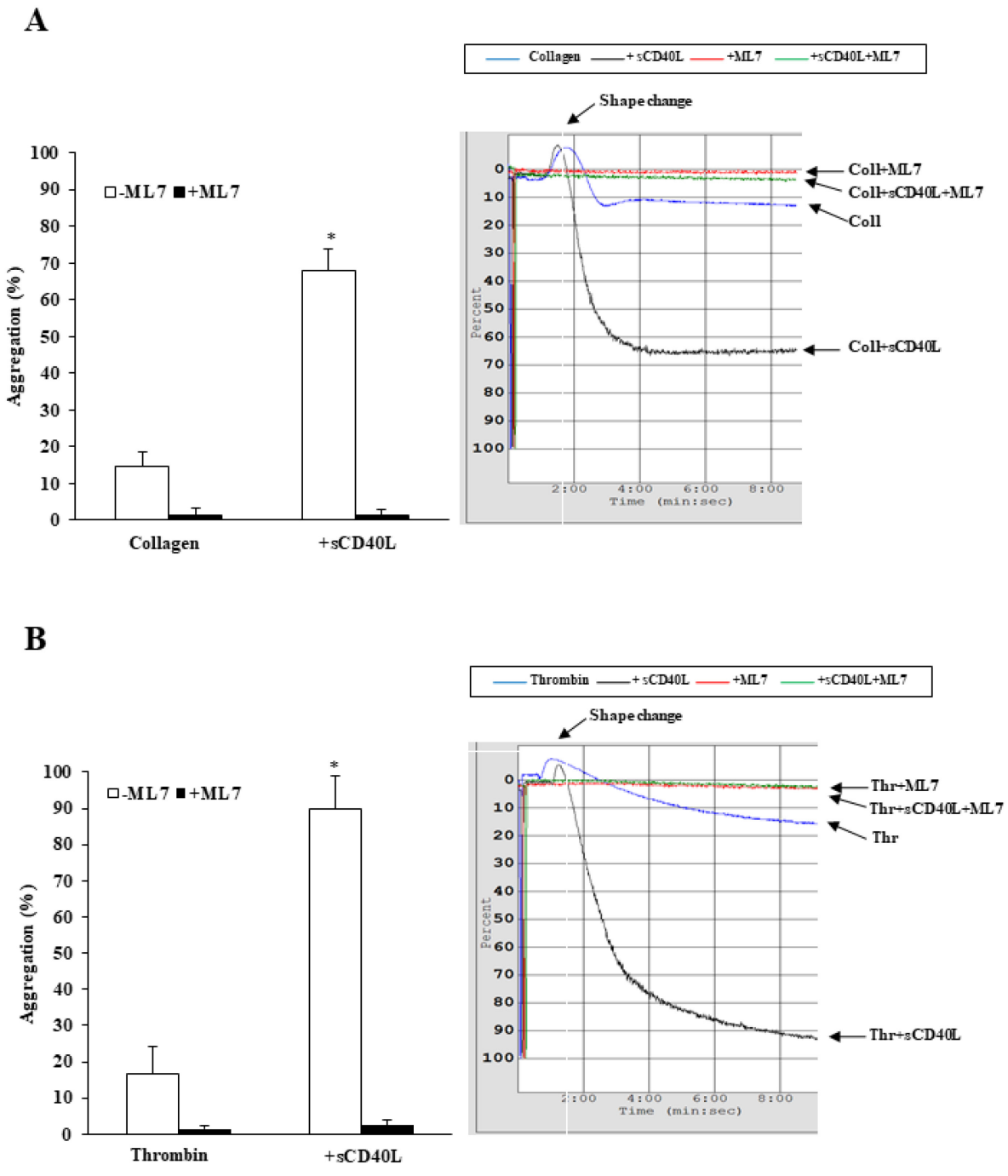
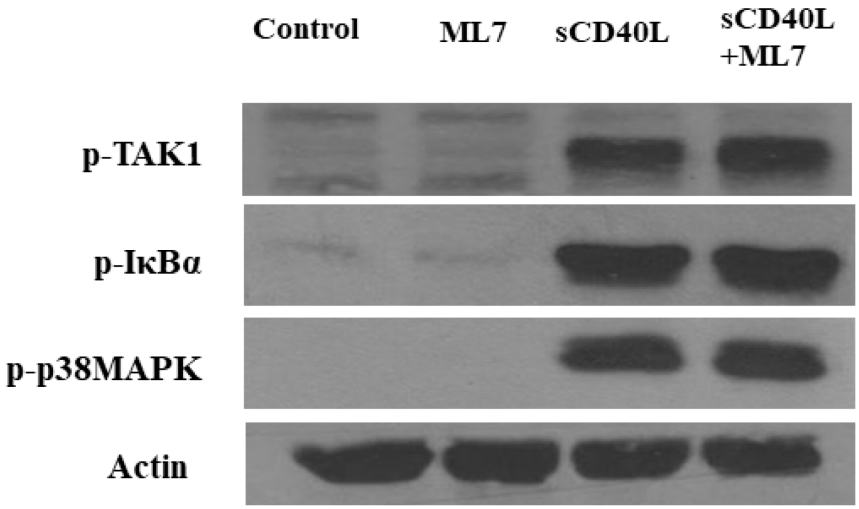
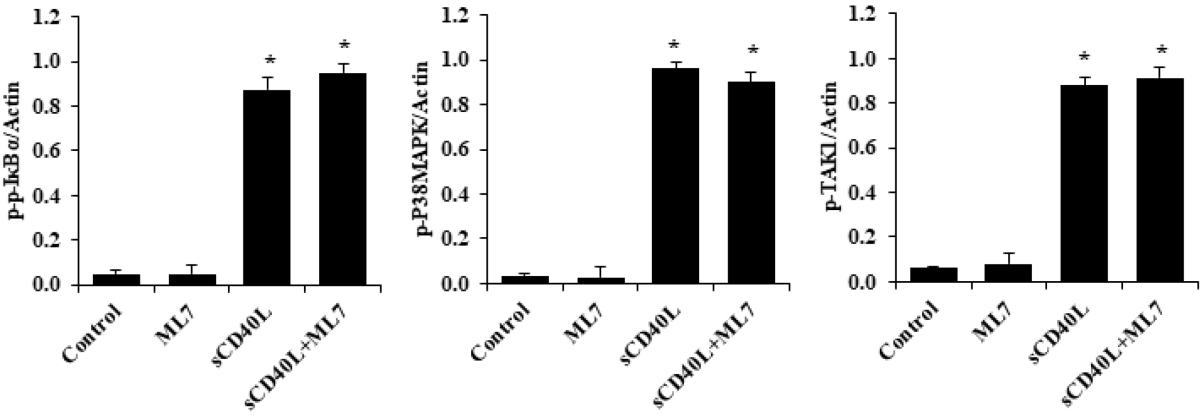

A
Collagen
+sCD40L
+ML7
+sCD40L+ML7
Shape change
-ML7 +ML7
Aggregation (%)
Coll+ML7
Coll+sCD40L+ML7
Coll
Coll+sCD40L
Percent
Time (min:sec)
Collagen
+sCD40L
B
Thrombin
+sCD40L
+ML7
+sCD40L+ML7
Shape change
-ML7 +ML7
Aggregation (%)
Thr+ML7
Thr+sCD40L+ML7
Thr
Thr+sCD40L
Percent
Time (min:sec)
Thrombin
+sCD40L

Figure 8


Control
ML7
sCD40L
sCD40L +ML7
p-TAK1
p-IκBα
p-p38MAPK
Actin


p-p-IκBα/Actin
p-P38MAPK/Actin
p-TAKI/Actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
ML7
sCD40L
sCD40L+ML7

Chapitre 6 : Discussion et Conclusion

1. Discussion

Les plaquettes jouent un rôle primordial dans la survenue des évènements cardiovasculaires. Elles modulent leurs propres réactions thrombo-inflammatoires par leur large répertoire de molécules pro-inflammatoires telles que les cytokines et chimiokines ainsi que des molécules pro-thrombotiques. Mon projet de doctorat gravite autour de l'une de ces molécules qui est le CD40L. Ainsi, lors de l'activation plaquettaire, le CD40L est exprimé à la surface des plaquettes et il est clivé en une forme soluble (sCD40L). D'ailleurs, 95% du sCD40L circulant provient des plaquettes activées (222, 260). Le niveau élevé de sCD40L circulant chez les patients est prédicteur fiable de risque des maladies cardiovasculaire. Effectivement, une association existe entre le niveau de sCD40L et l'apparition des complications cardiovasculaires dont l'athérosclérose et le SCA (292, 365). Le sCD40L module la fonction plaquettaire en interagissant avec trois de ces récepteurs plaquettaires, soit le CD40, $\alpha_{IIb}\beta_3$ et $\alpha_5\beta_1$ (191, 249, 278). L'interaction du sCD40L avec le CD40 plaquettaire entraine l'activation plaquettaire qui se traduit par leur changement de forme, l'expression de la P-séléctine, la sécrétion des α-granules et granules denses et l'activation de l'intégrine $\alpha_{IIb}\beta_3$ (278). Cependant, le sCD40L ne peut induire une agrégation plaquettaire; mais joue plutôt le rôle d'un amorceur *«primer».* Ainsi, il peut déclencher une agrégation plaquettaire en présence de doses sous-optimales d'agonistes plaquettaires comme la thrombine et le collagène. Au sein de notre laboratoire, on a démontré que le sCD40L amorce les plaquettes en activant la voie de signalisation CD40/TRAF2/Rac-1/p38 MAPK et induit l'agrégation plaquettaire en présence de doses sous-optimales de collagène (294). Les plaquettes traitées avec une forme de sCD40L muté qui ne reconnait pas le CD40 ($sCD40L^{R/Y}$) ou les plaquettes de souris $CD40^{-/-}$ sont incapables d'induire une telle réponse (294). Notre groupe a démontré aussi que le sCD40L amorce les plaquettes et les agrège en présence de doses sous-optimales d'agonistes *via* une voie de signalisation activant le NF-κB, une voie indépendante de p38 MAPK (295). Cela suggère que le NF-κB joue un rôle non-génomique dans l'amorçage des plaquettes. Dans les cellules nucléées, le CD40L active la voie de signalisation du NF-κB qui déclenche la transcription des gènes pro-inflammatoires impliqués dans l'initiation et la progression de différentes pathologies à caractère inflammatoire, dont l'athérosclérose. En effet, l'activation du NF-κB est impliquée dans le processus de

l'athérogénèse, incluant la formation des cellules spumeuses, l'inflammation vasculaire, la prolifération des CML, la calcification artérielle et la progression de la plaque (495). En 2002, une étude démontra que le NF-κB est exprimé au sein des plaquettes ainsi que son activation est induite par la dégradation de la protéine inhibitrice du NF-κB, le IκBα, en réponse à la thrombine. Cela engendra l'émergence de différentes études pour élucider la fonction d'un facteur de transcription au sein des plaquettes (496). Ainsi, le traitement des plaquettes avec des inhibiteurs de NF-κB dont le BAY 11-7082 altère la fonction plaquettaire en réponse à des agonistes plaquettaire comme le collagène et la thrombine (497, 498). Notre laboratoire a démontré que le sCD40L potentialise l'agrégation plaquettaire en présence de stimuli thrombotiques *via* l'activation du NF-κB (295).

Dès lors, le premier volet de mon projet de doctorat était d'élucider davantage la voie d'activation du NF-κB par le sCD40L, et cela en repérant en premier lieu les récepteurs de CD40L impliqués dans l'activation du NF-κB. Le CD40L interagit avec les cellules du système vasculaire à travers quatre récepteurs. Depuis la découverte du CD40L, le récepteur CD40 était considéré comme étant l'unique récepteur de CD40L. Cependant, en 2002, d'autres récepteurs de CD40L ont prouvé être capables de lier le CD40L, soit les intégrines $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ et $\alpha_M\beta_2$ (Mac-1) (191-193). Les plaquettes expriment trois récepteurs du CD40L qui sont CD40, $\alpha_{IIb}\beta_3$ et $\alpha_5\beta_1$ (191, 249, 278). Effectivement, comme on l'a mentionné auparavant, l'interaction CD40L/CD40 provoque l'activation plaquettaire en induisant le changement de forme et la sécrétion plaquettaire ainsi qu'elle potentialise l'agrégation plaquettaire. La liaison du CD40L à l'$\alpha_{IIb}\beta_3$ *via* le motif KGD induit la stabilisation des thrombi artériels in vivo ainsi que la signalisation «*outside-in*» de l'$\alpha_{IIb}\beta_3$ contribuant à l'activation plaquettaire (191). Une seule étude a rapporté que l'interaction CD40L/$\alpha_5\beta_1$ participe à l'activation des plaquettes (249). Dans cette étude, on a confirmé l'expression des trois récepteurs de CD40L sur les plaquettes, on n'a pas trouvé d'expression du Mac-1 puisqu'il est exprimé plutôt sur les lignées leucocytaires que sur les plaquettes. Nous avons aussi trouvé que l'interaction CD40L/CD40 participe à la sécrétion du sCD40L des plaquettes; ce processus de rétroaction positif suggère une amplification de l'effet d'amorçage des plaquettes par le CD40L *via* le CD40. L'amorçage des plaquettes contribue à l'hyperactivité plaquettaire, un phénomène observé chez les patients souffrant de coronaropathies avec un niveau élevé de sCD40L en circulation (292, 499, 500).

De plus, on a confirmé l'activation du NF-κB par le sCD40L en observant la phosphorylation de l'IκBα et la sous-unité du NF-κB, le p65. D'ailleurs, la phosphorylation de la protéine inhibitrice du NF-κB, le IκBα induit sa dégradation par le protéasome, ce qui permet à la sous-unité du NF-κB d'être phosphorylée (501). L'activation du NF-κB par le sCD40L pourra avoir un rôle dans la sécrétion plaquettaire. En effet, le sCD40L induit l'activation du IKKβ et son inhibition affecte la translocation de la P-séléctine des α-granules sur la surface plaquettaire (295). Un mécanisme pour la sécrétion plaquettaire est proposé par Karim et al. où l'activation du IKKβ phosphoryle le SNAP-23, une protéine appartenant au complexe SNARE dont l'activation favoriserait l'exocytose des granules en promouvant la fusion des vésicules à la membrane des plaquettes (502). D'ailleurs, des plaquettes de souris déficientes en IKKβ présentent une réduction de leur activation et sécrétion (503).

Afin d'identifier l'implication des récepteurs dans l'activation du NF-κB, on a eu recours à des antagonistes pharmacologiques, le 5C8 (antagoniste de l'interaction CD40L/CD40) le Reopro (l'abciximab, antagoniste de l'$\alpha_{IIb}\beta_3$) et le JBS5 (antagoniste de l'$\alpha_5\beta_1$). Ainsi, on n'observe aucune inhibition de la phosphorylation de l'IκBα et le p65 quand les plaquettes sont traitées avec le Reopro et le JBS5 et stimulées avec le sCD40L. Cependant, la phosphorylation de l'IκBα et le p65, et ainsi l'activité de NF-κB, est inhibée quand les plaquettes sont traitées avec le 5C8 et stimulées par le sCD40L. Cela indique que l'activation de NF-κB plaquettaire par le CD40L est exclusivement dépendante du CD40. Pour confirmer notre découverte, on a étudié l'activation du NF-κB par le sCD40L de souris (msCD40L) sur des plaquettes de souris $CD40^{-/-}$. Dans une étude précédente au sein de notre laboratoire, en utilisant un modèle de thrombose artérielle induite par lésion avec $FeCl_3$, on a démontré que l'infusion de msCD40L amorce les plaquettes et, par conséquent, exacerbe la formation de thrombose et l'infiltration leucocytaires chez les souris sauvages (WT), mais pas chez les souris $CD40^{-/-}$ (295). Au niveau de la présente étude, l'absence d'activation du NF-κB, dans les plaquettes de souris $CD40^{-/-}$ stimulées avec le msCD40L, concorde avec les résultats qu'on a trouvés en utilisant les inhibiteurs pharmacologiques ainsi que le modèle de thrombose de notre groupe (295) puisque l'activation du NF-κB est cruciale à l'amorçage plaquettaire et, par conséquent, l'exacerbation de la thrombose suite à l'infusion de msCD40L. Pour appuyer notre résultat dans la fonction plaquettaire, on a observé que les plaquettes traitées avec le 5C8 et le BAY-11 7082 (antagoniste

du NF-κB) sont incapables d'induire une potentialisation de l'agrégation plaquettaire induite par le sCD40L en présence de doses sous-optimales de thrombine. Par contre, le JBS5 n'a aucun impact sur la potentialisation d'agrégation induite par le CD40L, ce qui confirme que l'effet d'amorçage des plaquettes par le CD40L est indépendant de l'$\alpha_5\beta_1$. On ne peut observer l'effet du $\alpha_{IIb}\beta_3$ sur la potentialisation d'agrégation induite par le CD40L puisque le Reopro empêche la liaison du fibrinogène sur l'$\alpha_{IIb}\beta_3$, une étape primordiale dans l'agrégation plaquettaire (504). Ainsi, l'axe CD40/NF-κB joue un rôle crucial dans l'amorçage des plaquettes favorisant l'hyperactivité plaquettaire, un phénomène observé chez les patients souffrant de maladie cardiovasculaire. D'ailleurs, cette voie est impliquée dans le développement de différentes pathologies, dont les maladies auto-immunes, le cancer et l'athérosclérose (321, 322, 340, 341, 505). Notre résultat renforce le rôle pathologique de l'axe CD40/NF-κB au sein de l'organisme. Une étude menée par Kuijpers et al. rapportent que la potentialisation de l'agrégation plaquettaire des plaquettes par CD40L implique plutôt PI3Kβ et non pas CD40/NF-κB (300). Cependant, cette étude propose d'une part, qu'une déficience en CD40 mène à une augmentation de l'expression de l'$\alpha_{IIb}\beta_3$, ce qui contredit l'étude menée par Inwald et al. (278), le CD40L et d'une autre part, que l'expression de la P-séléctine et l'exposition de PtdSer ne sont pas affectées par la préincubation des plaquettes par le CD40L, ce qui contredit plusieurs autres études (278, 296, 297). De plus, au sein de notre laboratoire, on a démontré que des plaquettes incubées avec du sCD40L et stimulés avec une dose sous-optimale de collagène entraine une augmentation de l'expression de l'$\alpha_{IIb}\beta_3$ et P-séléctine (294). Cette différence de résultats peut être expliquée par le fait que Kuijpers et al. rapportent ces résultats en utilisant des plaquettes provenant de souris Apoe$^{-/-}$, un modèle de souris utilisé communément pour induire le développement spontané de lésions d'athérosclérose (506, 507). Les plaquettes de souris Apoe $^{-/-}$ sont hyper-réactives comparé au plaquettes WT par leur capacité à synthétisé d'avantage de TxA_2 (508) et une perte de l'activité des mécanismes inhibiteurs endogènes des plaquettes (509). Curieusement, une autre étude dont Kuijpers est un co-auteur soulève qu'une déficience en CD40 n'affecte pas l'activation plaquettaire. En effet, on observe que la stimulation des plaquettes de souris Apoe $^{-/-}$ CD40 $^{-/-}$ avec du collagène n'affecte pas l'agrégation plaquettaire comparée aux plaquettes de souris Apoe $^{-/-}$ (298). Cependant, une surexpression de l'$\alpha_{IIb}\beta_3$ sur les plaquettes de souris Apoe $^{-/-}$ CD40 $^{-/-}$ comme rapporté dans son étude (300) suggère qu'on assisterait à une augmentation de l'agrégation plaquettaire. Enfin, il serait intéressant de comparer l'activité plaquettaire des

souris Apoe $^{-/-}$ CD40 $^{-/-}$ aux plaquettes des souris WT et CD40 $^{-/-}$ afin d'observer davantage les modulations que peuvent engendrer la déficience en Apoe sur l'activité plaquettaire et son impact sur les plaquettes de souris CD40 $^{-/-}$.

Dans un second lieu, le *transforming growth factor-B (TGF-B)-activated Kinase* (TAK1) est un régulateur majeur de l'inflammation, et il émerge comme étant une cible thérapeutique attrayante pour les pathologies inflammatoires (510). Effectivement, une délétion du TAK1 prévient différentes pathologies dont l'inflammation rénale (511), l'hypertrophie des cardiomyocytes associée à la défaillance cardiaque (512) et la mort neuronale dans l'ischémie cérébrale (513). Dans les cellules nuclées, l'activation des récepteurs de la famille de TNF dont le CD40 induit l'activation du TAK1; ce dernier est capable à son tour d'activer différentes protéines, dont le NF-κB favorisant l'occurrence des pathologies inflammatoires (510). Ainsi, malgré que le TAK1 soit présent dans les plaquettes (514), sa fonction dans la signalisation CD40L/CD40/NF-κB est inconnue. Dès lors, notre première observation est que le sCD40L active le TAK1 dans les plaquettes. De plus, on a démontré que les antagonistes de TAK1 (ZOL et Takinib) inhibent IκBα de manière dose-dépendante. Ce résultat nous permet de déduire que le TAK1 est en aval de NF-κB au sein de plaquettes, et par conséquent, l'inhibition du TAK1 par le ZOL et Takinib bloque aussi la voie d'amorce du CD40L/CD40/NF-κB qui se traduit par une abolition de la potentialisation de l'agrégation plaquettaire induire par le sCD40L en présence de doses sous-optimales de thrombine. Dès lors, la voie CD40L/CD40/TAK1/NF-κB se définit comme étant une signalisation primordiale dans l'amorçage des plaquettes, ce qui peut participer à l'amplification des réactions inflammatoires. D'ailleurs, cette voie fut d'abord définie au niveau des cellules du carcinome; en effet, l'activation du CD40 exprimé sur les cellules cancéreuses induit le recrutement des TRAFs, et suite à cela, le déclenchement de la cascade TAK1/IKKβ/IκBα qui mène à la mobilisation du p65 afin de réguler l'activité transcriptionnelle de l'IRF-1 (*interferon regulator factor 1*) (515). De plus, TAK1 joue un rôle important dans la production des DRO et autres médiateurs pro-inflammatoires *via* la voie CD40L/CD40/NF-κB dans les CML (516).

En tout, ce premier volet me permet d'élucider davantage la voie CD40L/NF-κB au sein des plaquettes. Ainsi, l'activation du NF-κB par le CD40L est CD40 et TAK1 dépendante. Dès

lors, la voie CD40L/CD40/TAK1/NF-κB amorce et potentialise l'agrégation plaquettaire en présente de doses sous-optimales de stimuli thrombotiques. Ainsi, cette voie pourrait représenter une cible thérapeutique dans le traitement des pathologies thrombo-inflammatoire.

Le deuxième volet de ce projet de doctorat consiste à déceler l'impact du sCD40L sur l'efficacité de l'ASA. L'ASA est l'antiplaquettaire le plus communément utilisé pour la prévention et le traitement des thromboses artérielles cardio-cérébrales (374). Cependant, son efficacité est limitée; en effet, un large nombre de patients à haut risque de coronaropathie traités à l'ASA souffre toujours d'événements thrombotiques récurrents. Cette variabilité au niveau de la réponse est associée au phénomène de la résistance à l'ASA (400-406). La cause de cette résistance à l'ASA chez ces patients est plurifactorielle incluant la pharmacocinétique de l'ASA, les interactions médicamenteuses et le polymorphisme du COX-1. De plus, ces patients résistant à l'ASA peuvent présenter des plaquettes hyperactives dues à la présence d'un niveau élevé de molécules thrombo-inflammatoires qui agissent en tant qu'amorceurs(517, 518). D'ailleurs, une étude publiée par Blair et al. démontre que les amorceurs circulant, l'IGF-1 (*insulin-like growth factor-1*) et la thrombopoïétine contribuent au phénomène de résistance à l'ASA en reversant l'effet de l'antiplaquettaire de manière PI3K-dépendante et PI3K-indépendante (429). Ainsi, comme on l'a présenté dans le premier volet de cette étude, le sCD40L est un amorceur plaquettaire capable de potentialiser l'agrégation plaquettaire en présence de doses sous-optimales d'agonistes. Par conséquent, un taux sérique élevé de sCD40L chez les patients coronariens est associé à un risque élevé d'évènements thrombotiques. Cependant l'implication du sCD40L sur l'efficacité de l'ASA demeure inconnue.

Afin d'investiguer l'effet de l'ASA sur la fonction d'amorçage du sCD40L sur les plaquettes, on a d'abord évalué la potentialisation de l'agrégation plaquettaire induite par le sCD40L avec des plaquettes traitées ou non avec l'ASA en présence de doses sous-optimales de thrombine ou de collagène. Ainsi, on a observé que l'ASA renverse complètement l'effet de potentialisation de l'agrégation plaquettaire déclenché par le sCD40L en réponse à des doses sous-optimales de collagène. Par contre, l'ASA réduit partiellement, mais significativement, cet effet quand les plaquettes traitées avec le sCD40L sont stimulées avec des doses sous-optimales de thrombine. Parallèlement, le sCD40L est capable de diminuer l'effet antiplaquettaire de

l'ASA de manière significative. Cette variabilité entre les deux agonistes sur l'effet de l'ASA sur les plaquettes traitées avec le sCD40L s'explique par le fait que l'action proagrégante du collagène dépend de la présence du TxA_2; or la thrombine, étant un agoniste puissant de plaquettes, peut provoquer l'agrégation des plaquettes de manière indépendante de TxA_2 (519-521). En effet, l'action de la thrombine et le collagène sur l'activation plaquettaire consiste majoritairement par l'activation de la phospholipase C (PLC) *via* les récepteurs PARs et GPVI respectivement. Ainsi, l'activation de la PLC par ces deux agonistes entraine l'hydrolyse de phosphatidylinositol ce qui conduit à l'activation des isoformes des PKC et la libération du calcium intracellulaire *via* la génération du DAG et l'IP3 respectivement. Pour mieux visualiser ces voies de signalisations, différents schémas dans le chapitre 1 de la thèse décrivent l'activation plaquettaire *via* le PLC (ex : Figure 1.10). Ainsi, la thrombine est considérée comme un agoniste puissant, car parmi les agonistes plaquettaires qui agissent en activant la phospholipase C (PLC) dont le collagène, la thrombine est le plus efficace a entrainé l'hydrolyse du phosphatidylinositol et ainsi, la plus rapide à induire une augmentation importante et exponentielle de calcium cytosolique, cet évènement est primordial puisque différentes cascades menant à l'activation plaquettaire dépendent du calcium dont la sécrétion des granules (522). De plus, la thrombine est capable d'inhiber l'activité de l'adénylate cyclase favorisant davantage l'activation plaquettaire *via* Gi, une voie qui est absente par le collagène. Ainsi, la thrombine n'est pas de dépendante du TXA_2 afin d'induire une agrégation maximale, ce qui n'est pas le cas du collagène, qui comme l'ADP, dépend de la biosynthèse et la sécrétion de la TXA_2 pour achever une telle agrégation (522). Enfin, l'activation plaquettaire est affecté par l'ASA quand les plaquettes sont stimulées avec le collagène, mais non avec la thrombine (523). En effet, malgré la double thérapie impliquant le clopidogrel et l'ASA, un large nombre de patients atteints de SCA présentent des événements ischémiques récurrents majeurs dus à des marqueurs d'activation de la thrombine qui demeurent élevés après l'occurrence d'un SCA (524, 525). Ceci suggère qu'il existe une activation plaquettaire maintenue par un mécanisme médié par la thrombine (526).

Hormis l'effet inhibiteur de l'ASA dans la potentialisation induite par le sCD40L dans l'agrégation plaquettaire en présence de collagène, on observe que l'ASA engendrait aussi l'inhibition du changement de forme des plaquettes stimulées par le collagène. Cet effet n'est pas observé quand l'agrégation plaquettaire est induite par la thrombine. Pour investiguer

davantage l'effet de l'ASA sur le changement de forme des plaquettes, on s'est intéressé à l'activation de la MLC. Le changement de forme des plaquettes est un événement critique pour l'étalement et l'adhésion plaquettaire qui requiert le remodelage dynamique du cytosquelette d'actine. La protéine régulatrice principale de la fonction de l'actine dans les plaquettes est la myosine IIa; ainsi, l'activation de la MLC est cruciale pour l'interaction de la myosine aux filaments d'actine nécessaire au changement de forme plaquettaire (126, 527, 528). D'ailleurs la MLC est une cible potentielle pour le traitement des pathologies inflammatoires (529). Dès lors, on observe dans cette étude que l'ASA inhibe la phosphorylation de la MLC des plaquettes stimulées avec le collagène, mais pas avec la thrombine, qu'il s'agisse de plaquettes traitées avec du sCD40L ou non. Ce résultat nous indique que l'ASA prévient exclusivement l'action du collagène sur les plaquettes *via* l'inhibition de la MLC. Ce résultat est confirmé avec l'utilisation du ML7 (antagoniste de la MLC kinase); ainsi, le ML7 est capable d'inhiber l'action de potentialisation du sCD40L en réponse au collagène et à la thrombine. Cette inhibition est indépendante de la voie de signalisation de sCD40L puisque le ML7 n'affecte pas la phosphorylation des protéines TAK1, NF-κB et p38 MAPK. Rappelons qu'au sein de notre laboratoire, on a décelé deux voies d'amorçage, soit l'axe CD40/TAK1/NF-κB et Rac1/p38 MAPK (294, 295, 530). Ainsi, l'ASA agit sur l'effet de potentialisation de sCD40L en agissant sur l'agoniste plaquettaire permettant l'induction de l'agrégation des plaquettes amorcées. L'action de MLC étant nécessaire à l'activité de l'agoniste, l'ASA prévient l'action du collagène *via* l'inhibition de la MLC; cependant, l'ASA n'affecte pas l'activation de la MLC des plaquettes stimulées avec la thrombine, qui se traduit par un maintien du changement de forme et de l'effet de potentialisation du sCD40L dans l'agrégation plaquettaire. La capacité de la thrombine à induire l'activation de la MLC en présence de l'ASA suggère que la thrombine possède un mécanisme de changement de forme qui peut être indépendant de TxA_2.

Au sein de cette étude, on a observé aussi que le sCD40L est capable d'amplifier la sécrétion de TxA_2 en présence de doses sous-optimales d'agonistes. La production du TxA_2 est abolie une fois que l'on traite les plaquettes avec l'ASA. Ainsi, hormis la capacité du sCD40L à favoriser la sécrétion plaquettaire (278), elle peut augmenter la génération du TxA_2. D'ailleurs, similairement au sCD40L, d'autres amorceurs plaquettaires sont capables d'amplifier la production du TxA_2 (429). Dès lors, malgré que le pretraitement avec l'ASA des plaquettes

incubées avec le sCD40L et des doses d'agonistes est abolie leur production du TxA_2, le sCD40L maintient sa capacité à potentialiser l'agrégation plaquettaire en présence de doses sous-optimales de thrombine. Ce résultat démontre que la signalisation d'amorçage induite par le sCD40L est indépendante du TxA_2, ce qui peut expliquer la résistance à l'ASA des plaquettes suite à leur incubation avec le sCD40L; d'ailleurs, un des mécanismes de résistance à l'ASA est les voies alternatives d'activation plaquettaire qui sont indépendantes du TxA_2 (531). De plus, nos résultats suggèrent que l'ASA n'impacte pas l'activation des voies de signalisation du sCD40L. En effet, l'ASA n'affecte pas la phosphorylation des protéines impliquées dans la signalisation d'amorçage du sCD40L, TAK1, NF-κB, et p38 MAPK, quand ces plaquettes sont traitées à l'ASA et stimulées avec du sCD40L. Ainsi, l'ASA agit de manière indirecte sur la potentialisation du sCD40L en agissant sur les agonistes responsables de l'induction des plaquettes amorcées. En effet, l'action de l'ASA sur l'effet de potentialisation dépend de la dépendance de l'agoniste au TxA_2. En effet, on observe que la potentialisation de l'agrégation plaquettaire induite par le sCD40L est inhibée en présence du collagène dont l'action est dépendante du TxA_2, ce qui n'est pas le cas avec la thrombine qui peut provoquer une agrégation plaquettaire indépendamment du TxA_2. Ainsi, dans un contexte plus clinique, nos résultats suggèrent que les patients qui suivent un traitement antiplaquettaire consistant en des antagonistes de TxA_2 comme l'ASA ne sont pas protégés de la fonction d'amorçage du sCD40L sur les plaquettes.

Ainsi, dans ce deuxième volet de mon projet, nous avons trouvé que l'ASA n'affecte pas la signalisation du sCD40L plaquettaire suite au maintien de la phosphorylation du TAK1, NF-κB, et p38 MAPK. Cependant, le sCD40L a potentialisé la production de TxA_2, ainsi que l'agrégation plaquettaire, *via* un mécanisme impliquant le changement de forme plaquettaire et la phosphorylation de la MLC. Dès lors, le taux élevé de sCD40L sérique peut faire varier l'efficacité de l'ASA et contribuer au phénomène de résistance chez les patients souffrant de coronaropathies. Ainsi, le ciblage de l'axe du CD40L dans les plaquettes peut avoir un potentiel thérapeutique chez les patients présentant des taux élevés de CD40L et qui ne répondent pas ou moins à l'ASA.

2. Conclusion

Ce projet m'a permis de déterminer que l'amorçage des plaquettes par le CD40L *via* l'activation de NF-κB dépend du récepteur CD40 et du TAK1. Ainsi, l'axe sCD40L/CD40/TAK1/NF-κB potentialise l'activation et l'agrégation en réponse à des stimuli thrombotiques, ce qui peut favoriser l'occurrence d'événements athéro-thrombotiques chez les patients coronariens. De plus, étant donné que l'ASA n'a pas d'effet sur la signalisation d'amorçage du sCD40L, le ciblage de cet axe dans les plaquettes peut avoir un potentiel thérapeutique chez les patients coronariens présentant des taux élevés de sCD40L et réagissant pas ou moins à l'ASA. Ainsi, nos études précédentes et les résultats de ce projet me permet de proposer un schéma (Figure 5.1) résumant la signalisation d'amorçage de la voie sCD40L/CD40 et ses conséquences dans les plaquettes.

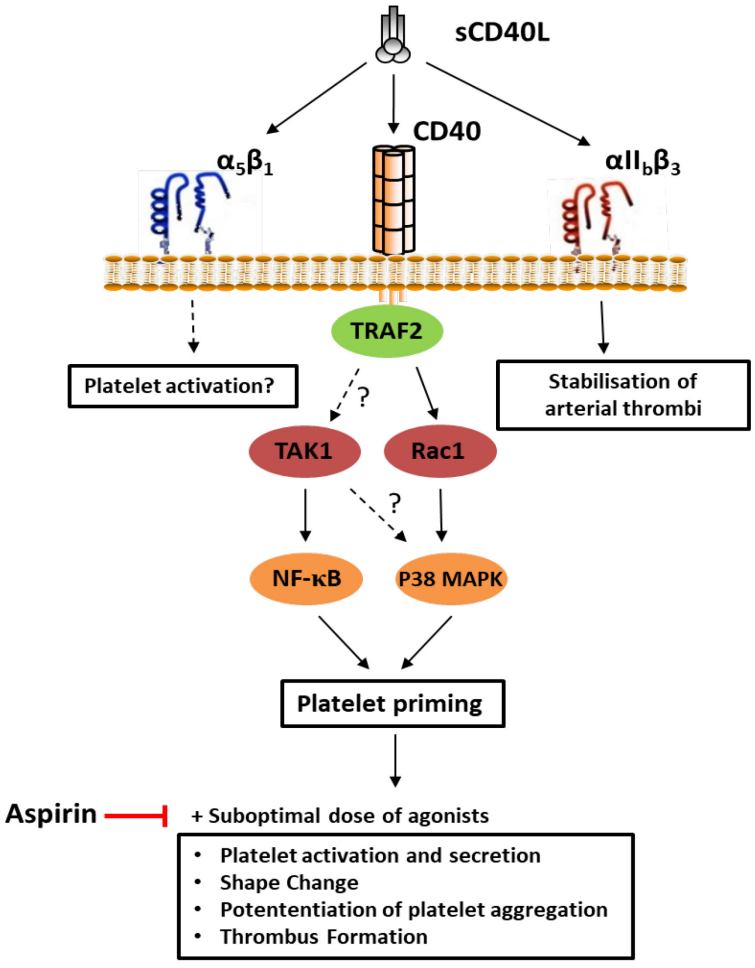


Figure 5.1: La signalisation d'amorçage de la voie sCD40L/CD40

3. Perspectives

La présence de médiateurs thrombo-inflammatoires dans la circulation sanguine des patients souffrant des maladies cardiovasculaires amplifie l'athérosclérose et la thrombose, et par conséquent, les complications cliniques qui en résultent dont le SCA et l'IM (493). Le sCD40L fait partie des principaux médiateurs thrombo-inflammatoires qui émergent comme étant des prédicteurs fiables des maladies cardiovasculaires (213, 267, 494) et particulièrement chez les patients souffrant de diabète (269, 287, 288), hypercholestérolémie (289, 290), athérosclérose (291) et SCA (270, 292, 293). On a démontré, au sein de notre laboratoire, que le sCD40L amorce les plaquettes et potentialise l'agrégation plaquettaire en présence de doses sous-optimales d'agonistes. Les voies d'amorçages de sCD40L impliquent l'axe CD40/TAK1/NF-κB et l'axe CD40/Rac-1/p38 MAPK (294, 295, 530). Ainsi, il serait intéressant d'investiguer la fonction plaquettaire chez les patients souffrant de coronaropathie en étudiant la signalisation d'amorçage induite par le sCD40L, l'activation, la sécrétion et l'agrégation plaquettaire dans le but de déterminer une corrélation entre le niveau de sCD40L et la fonction plaquettaire chez les patients souffrant de coronaropathie. De plus, nos résultats révèlent que l'ASA n'a pas d'effet sur la signalisation du sCD40L; ainsi, l'investigation de la fonction plaquettaire chez des patients coronariens sous traitements antiplaquettaires, et particulièrement à l'ASA, nous permettrait déterminer une corrélation entre le sCD40L et la résistance à l'ASA.

L'identification d'une cible thérapeutique afin de prévenir l'effet de l'axe CD40L/CD40 dans l'exacerbation des maladies cardiovasculaires est une poursuite attrayante. Cependant, l'axe CD40L/CD40 est présent dans une panoplie de lignées cellulaires et joue un rôle primordial dans des fonctions physiologiques dont l'immunité et l'apoptose cellulaire (187, 223). Par conséquence, le ciblage de l'axe CD40L/CD40 pourrait avoir des effets délétères sur le système immunitaire comme ceux observés chez les patients atteints de HIGM (532) ainsi que sur l'arsenal de l'organisme à combattre les cellules cancéreuses (533). Dès lors, le développement d'outils thérapeutiques devrait prendre en considération ces effets et viser une signalisation spécifique de l'axe CD40L/CD40 plaquettaire. Ainsi, l'activation du CD40 par le CD40L induit le recrutement des TRAFs, (TRAF1-6) qui permet de déclencher l'activation des voies de signalisation en aval du CD40 (208). Au sein de notre laboratoire, on a démontré que

les plaquettes contiennent le TRAF1, TRAF2, TRAF6 et un niveau négligeable de TRAF3. De plus, on a trouvé que l'activation du CD40 induit l'association du TRAF2 exclusivement suite à une stimulation du sCD40L au sein des plaquettes (294). De ce fait, l'investigation de la signalisation CD40/TRAF2 à travers l'usage d'antagonistes pharmacologiques ou de modèles de souris knock-out déficientes en TRAF2 ($TRAF2^{-/-}$) nous permettrait d'élucider davantage l'impact du TRAF2 sur la fonction d'amorçage de l'axe CD40L/CD40 et d'identifier une nouvelle cible thérapeutique pour prévenir l'effet thrombo-inflammatoire de l'axe CD40L/CD40 plaquettaire sans affecter les autres fonctions de l'organisme.

L'usage de souris knockout nous permettrait de renforcer nos résultats obtenus avec des plaquettes humaines traitées avec des antagonistes pharmacologiques. On a proposé, dans le paragraphe précédent, le recours aux souris knock-out $TRAF2^{-/-}$ afin de déterminer l'implication du TRAF2 dans la fonction de l'axe CD40L/CD40 plaquettaire. Au sein de mon projet de doctorat, on a établi l'importance de l'axe CD40/TAK1/NF-κB dans la signalisation d'amorçage du CD40L, en utilisant des antagonistes pharmacologiques pour inhiber le TAK1 (ZOL et Takinib) et le NF-κB (BAY 11-7082) (530). Un modèle de souris knockout pour ses protéines renforcerait l'ampleur de la voie CD40/TAK1/NF-κB au niveau signalétique ainsi que dans l'exacerbation de la formation de thombus. Cependant, la déficience en TAK1, IKK ou NF-κB entraine la mort embryonnaire des souris due au rôle critique du NF-κB dans l'embryogenèse (534). Afin de contourner le problème de la létalité embryonnaire, on pourrait induire une mutagenèse conditionnelle chez des souris adultes (système Cre/loxP) (502, 535-538). De plus, un autre type de souris knock-out qui sera attrayant à utiliser est la souris $LDLr^{-/-}/Tg(ApoB^{+/+})$. Ces souris développent des lésions athérosclérotiques large et riche en lipides, une réduction de la fonction vasculaire et une augmentation de l'adhésion leucocytaire sur l'endothélium comparé à des souris WT (539). Au sein de notre laboratoire, on a observé, dans un modèle de thrombose artérielle induite par lésion avec $FeCl_3$, que l'infusion de sCD40L de souris exacerbe la formation de thrombose et l'infiltration leucocytaires chez les souris sauvages (WT), mais pas chez les souris $CD40^{-/-}$ (294). Dès lors, il serait intéressant d'investiguer chez les souris $LDLr^{-/-}/Tg(ApoB^{+/+})$ ayant subi une lésion vasculaire, l'effet amorcé du sCD40L sur la fonction de leurs plaquettes ainsi que l'impact de l'inhibition de l'axe CD40L/CD40 sur l'athéro-thrombose.

Signification clinique

Pour donner à mon projet une signification clinique, nos résultats suggèrent que les patients sous traitement antiplaquettaire bloquant la formation de TxA_2 ne sont pas totalement protégés de l'impact d'amorçage du sCD40L sur les plaquettes, suggérant qu'un taux élevé de sCD40L pourrait réduire l'efficacité des traitements antiplaquettaires. Ainsi, révéler les mécanismes d'amorçage de l'axe CD40L / CD40 dans la fonction plaquettaire pourrait aider à identifier des cibles moléculaires dans ses voies de signalisation susceptibles de devenir des traitements préventifs innovants chez les patients coronariens présentant des taux circulants élevés de sCD40L. Le principal résultat clinique consiste à améliorer la gestion des événements athéro-thrombotiques chez les patients atteints de coronaropathie qui ne répondent pas ou peu aux traitements antiplaquettaires conventionnels. Cela apportera une valeur significative au système de santé au-delà des programmes de prévention actuels et des thérapies traditionnelles existantes en coronaropathie. En effet, des études prospectives peuvent être planifiées chez des patients présentant un syndrome métabolique à risque élevé de coronaropathie et sachant que leur taux de sCD40L circulant est élevé. En outre, il serait également bénéfique de trouver un sous-groupe de patients qui ne répondent pas ou peu aux traitements antiplaquettaires et dont les plaquettes peuvent être manipulées in vitro avec un traitement expérimental interférant avec l'axe sCD40L / CD40. Le récepteur CD40, sa protéine adaptatrice TRAF2 et les effecteurs aval TAK1, p38MAPK et NF-KB sont impliqués dans l'axe sCD40L / CD40. Cependant, les voies spécifiques qui peuvent être ciblées pharmacologiquement dans les plaquettes, sans affecter les autres fonctions physiologiques, restent à identifier et à caractériser dans des études ultérieures.

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